THE UNIVERSITY *of* EDINBURGH

The Role of *Grb2* in Growth and Differentiation of Embryonic Stem Cells

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Declaration

This thesis has been composed by the candidate.

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Abstract

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst stage embryo. They exhibit unlimited proliferation in culture and have the ability to differentiate into all three germ layers of the developing organism, a property defined as pluripotency. Previously it was reported that growth factor-bound protein 2 (*Grb2*) is required for differentiation of the epiblast, the embryonic tissue that harbours the pluripotent founder cells of the foetus.

GRB2 is an adapter protein involved in the activation of the mitogen-activated protein kinase (MAPK) pathway in response to extracellular signals. It has also been implicated in the activation of the phosphoinositol-3-kinase (PI3K) pathway in response to fibroblast growth factor (FGF) signaling.

The work presented in this thesis examines the role of *Grb2* in ES cells and describes previously unreported contributions of this adaptor protein in regulating ES cell growth and differentiation.

It has been previously been shown by others that *Grb2* deficient (*Grb2*$^{-/-}$) cells grow relatively normally in ES growth medium containing serum. However, in serum free conditions (N2B27 medium) in this project, proliferation of *Grb2*$^{-/-}$ cells is reduced compared with wild type and "restored" *Grb2*$^{-/-}$ cells stably expressing a *Grb2* cDNA mini gene.

Under serum free conditions, *Grb2*$^{-/-}$ cells grow in tight, refractive colonies. Nanog expression was uniformly upregulated, in contrast to the heterogeneous pattern reported in serum-based medium. Colony expansion on the substratum appears to be compromised, although there is no apparent defect in the initial attachment of *Grb2*$^{-/-}$ cells. Cell cycle analysis indicates that the slower growth of *Grb2*$^{-/-}$ cells in serum free medium could be due to lengthening of the G1 phase of the ES cell cycle.

In an attempt to identify the signalling deficiency responsible for the growth defect of *Grb2*$^{-/-}$ cells, MAPK activation was restored by two methods, PMA a ligand that bypasses the requirement for *Grb2*, and Raf-ER, a conditionally regulated component of the MAPK pathway that acts downstream of *Grb2* in the MAPK pathway. Although both approaches increased MAPK signalling they were unable to rescue the growth defect. This suggests that MAPK is not required or alone is not

sufficient. Inhibition of Glycogen synthase kinase 3 β (GSK3β) is known to augment growth of ES cells under MAPK inhibition. Surprisingly, GSK3β inhibition did not enhance *Grb2*$^{-/-}$ cell growth. Under GSK3β inhibition, *Grb2*$^{-/-}$ ES cells fail to thrive. It is hypothesised that under these conditions cells undergo hyper-self-renewal at the cost of growth.

Grb2$^{-/-}$ ES cells are reported to exhibit limited differentiation potential. To examine the potency of *Grb2*$^{-/-}$ cells, these cells were subjected to embryoid body (EB) and monolayer differentiation. Analysis of EBs showed a loss of *Gata4*, *Gata6* and endoderm marker gene expression. However, markers of ectoderm (*Sox1*, *Pax6*, MAP2), the late epiblast/nascent mesoderm (*Brachyury)* and markers associated with gastrulation (*Twist* and *Snail*) were expressed. Outgrowths of morphologically and immunohistochemically identifiable neuronal cells confirmed differentiation of ectodermal cell types, indicating *Grb2* is not required for neuronal differentiation. However, beating cardiomyocytes could not be identified in *Grb2*$^{-/-}$ EBs, though readily found in restored *Grb2*$^{-/-}$ cells expressing the *Grb2* cDNA. This suggests that there is an essential role for *Grb2* in the mesoderm/cardiomyocyte differentiation pathway. This may be due to a defect in GATA factor expression since these factors are essential for cardiogenesis.

In serum-free monolayer differentiation, *Grb2*$^{-/-}$ cells formed neuronal cells. Additional inhibition of the MAPK pathway using a small chemical inhibitor failed to prevent this differentiation. However, biochemical analysis of the cells indicates that this occurs when ERK activation is very low, indicating differentiation was not MAPK-independent.

Grb2 mediates FGF-MAPK induced exit from the naïve ground state. These data suggest a *Grb2*-independent pathway can also facilitate this transition. *Grb2* is dispensable for differentiation in to some lineages. However as differentiation of *Grb2*$^{-/-}$ ES cells is restricted, this indicates *Grb2* is required for true pluripotency.

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Abbreviations

4OHT	4-Hydroxy-tamoxifen
ADE	Anterior Definitive Endoderm
AP	Alkaline Phosphatase
BIO	6-Bromoindirubin-3'-oxime
BMP4	Bone Morphogenetic Protein 4
CAM	Cell Adhesion Molecule
Cdx2	Caudal Related Homeobox
ChIP	Chromatin Immunoprecipitation
CHR	CHR99021
cm	Centimetre
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl Sulfoxide
EB	Embryoid Body
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to Mesenchyme Transition
EpiSC	Epiblast Stem Cell
ERK	Extracellular-Related Kinase
ES cell	Embryonic Stem Cell
FAK	Focal Adhesion Kinase
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FRS	FGF-Receptor Substrate
Gab	*Grb2*-associated Binding Protein
GEF	Guanine Exchange Factor
GMEM	Glasgow Minimal Medium
Grb2	Growth Receptor Bound Protein 2
GSK3β	Glycogen Synthase Kinase 3 β
H_2O	Water
HSPG	Heparan Sulfate Proteoglycan
ICM	Inner Cell Mass
Id	Inhibitor of Differentiation
IGF	Insulin-like Growth Factor
IL-6	Interleukin 6

iPS cell	Induced Pluripotent Stem Cell
IRS	Insulin Receptor Substrate
JAK	Janus Kinase
JNK	Jun N-Terminal Kinase
Klf4	Kruppel-like Factor
LB	Lysogeny Broth
LIF	Leukaemia Inhibitory Factor
LIF	Leukaemia Inhibitory Factor
MAPK	Mitogen-Activated Protein Kinase
MEF	Murine Embryonic Fibroblast
MEK	MAPk/ERK Kinase
Mins	Minutes
MKP3	MAPK Phosphatase 3
ml	Millilitre
MPC	Mesendoderm Progenitor Cell
mTOR	Mammalian Target of Rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N2B27-LB	N2B27 Defined Medium + LIF and BMP4
O/N	Overnight
^{o}C	Degrees Celsius
PBS	Phosphate Buffered Saline
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD03	PD0325901
PDK1	Phospatidylinositol-3-phosphate Dependent Kinase 1
PE	Parietal Endoderm
PI3K	Phosphoinositol 3 Kinase
PKA	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
PtdIns	Phosphatidylinositol
PTEN	Phosphatase/Tensin Homolog
RA	Retinoic Acid
RAOSMC	Rat Aortic Smooth Muscle Cell
Rb	Retinoblastoma Protein
ROCK	Rho Kinase
rpm	Revolutions Per Minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
RTK	Receptor Tyrosine Kinase
SCID	Severe Comined Immunodeficient
SCNT	Somatic Cell Nuclear Transfer
SDS	Sodium Dodecyl Sulfate
SH2	Src Homology 2
SHH	Sonic Hedgehog
SHIP2	SH2-Containing Inositol-5'-Phosphatase 2
SOB	Super Optimal Broth
SOCS	Supressor of Cytokine Signalling
SOS	Son of Sevenless

Sox	Sex Determining Region Y-box
SRF	Serum Response Factor
STAT	Signal Transducers and Activators
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
Tcl1	T-Cell Lymphoma Protein 1
TGFβ	Transforming Growth Factor β
VE	Visceral Endoderm
VEGF	Vascular Endothelial Growth Factor
WASp	Wiskott-Aldrich Syndrome Protein
XEN	Extraembryonic Endoderm

CHAPTER 1
Introduction

1.1 Pluripotent Embryonic Cells

Pluripotency is a property that is defined as the potential of a cell to develop into the differentiated cell types that make up the tissues of an organism. There are many types of pluripotent cell that share specific characteristics. These include the ability to expand indefinitely in culture, maintenance of self-renewal and the capacity to differentiate into more specialised cell types. A discussion of different types of pluripotent cell follows.

1.1.1 Embryonic Carcinoma Cells

Murine embryos can form teratocarcinomas when injected subcutaneously (Solter *et al*., 1970). A teratoma is a tumour mass constituted of a random assortment of differentiated cell types, encompassing all three germ layers; ectoderm, mesoderm and endoderm. A teratocarcinoma is a malignant form of teratoma derived from pluripotent germ cells. Ectopic transplantation of embryos at embryonic stage E7.0 (Figure 1.1) results in the formation of teratocarcinomas at low frequency (Solter *et al*., 1970). Later, Diwan and Stevens (1976) refined this research to show the origin of these tumours was the epiblast. The undifferentiated cells present in these tumours can also be expanded in culture to create cell lines (Finch and Ephrussi, 1967). Martin and Evans (1975) demonstrated that a fibroblast feeder layer aided maintenance and expansion of these cells and named them embryonal carcinoma (EC) cells.

EC cells retain an ability to differentiate upon transplantation into a syngeneic murine host (Damjanov *et al*, 1982) and injection into blastocyst embryos results in contribution to many lineages of the embryo, excluding the germ line (Brinster 1974; Rappolee *et al*., 1994). The inability of these cells to form germ cells may be explained by the typically abnormal karyotype exhibited by EC cells and the consequent termination of meiosis (Bradley *et al*., 1984). This early research led to the hypothesis that pluripotent cells could be derived directly from the embryo.

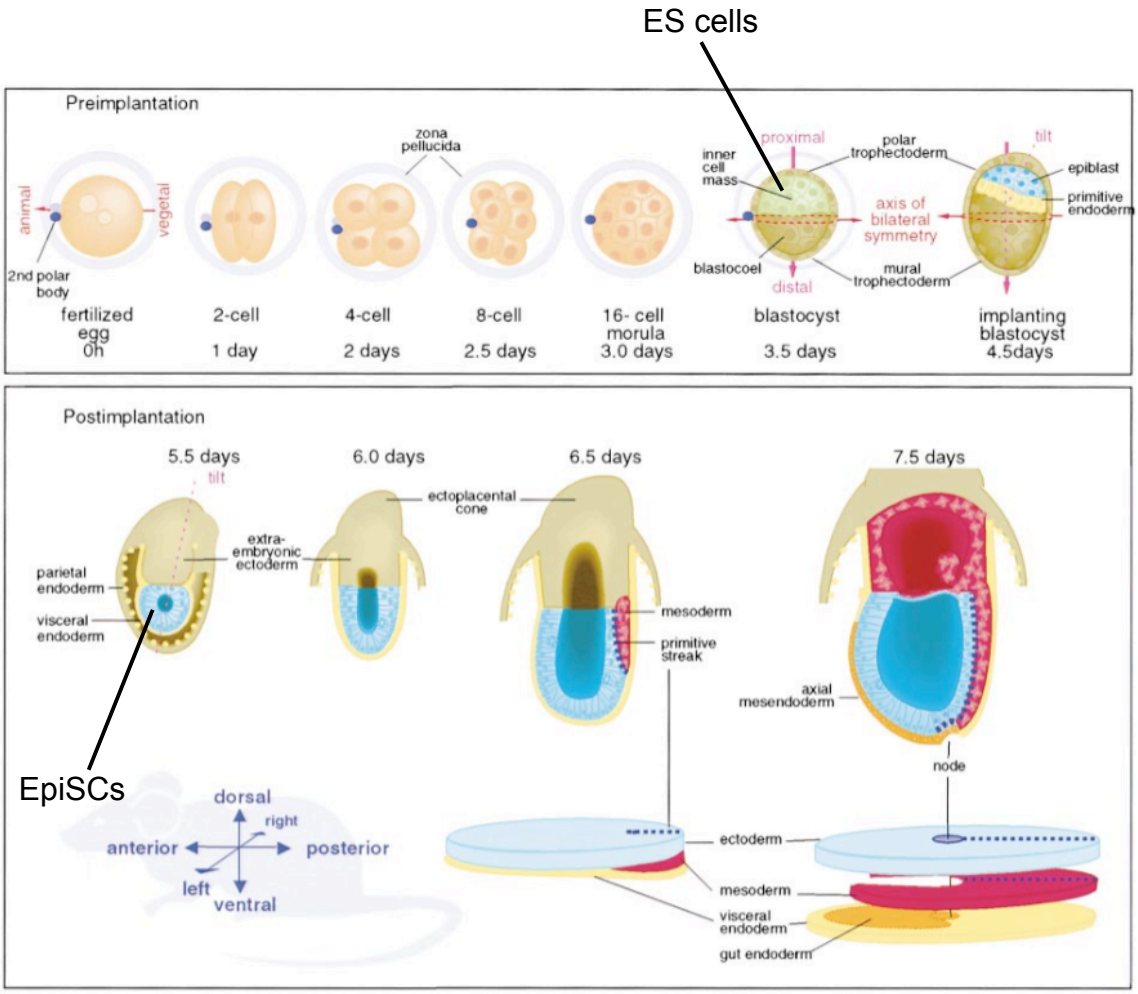


Figure 1.1 Early mouse development. Figure directly copied from Beddington and Robertson (1999) with some additional annotation.

1.1.2 Embryonic Stem Cells

Embryonic stem (ES) cells are derived from pre-implantation embryos. Upon plating the inner cell mass (ICM) of an embryonic stage E3.5 blastocyst onto a fibroblast feeder layer in the presence of foetal calf serum, a pluripotent cell population can be expanded (Evans and Kaufman, 1981; Martin, 1981). Like EC cells, ES cells proliferate indefinitely and differentiate into all three germ layers. ES cells have the greatest potency of all embryonic cell types and represent a transient population of cells that appear in the inner cell mass of the pre-implantation blastocyst.

Injection of ES cells into pre-implantation blastocysts show their capacity to contribute to chimeric mice is greater than that of EC cells and without tissue bias (Brinster *et al*, 1974). As their karyotype is diploid and stable, ES cells are also able to contribute to the germ line (Bradley *et al*., 1984) and pass the most stringent test of pluripotency: tetraploid complementation (Nagy *et al*., 1993). In this technique, tetraploid embryos are created upon fusion of late two-cell-stage embryos to one-cell-stage embryos. These embryos develop into blastocysts, albeit lacking an ICM. ES cells are injected into the blastocoel and, providing they are of sufficient quality, go on to form every tissue of the developing foetus.

The ability of ES cells to contribute to the germ line coupled with their amenability to genetic manipulation has enabled research into the role of genes both in development and the adult organism.

The availability of ES cells presents an opportunity to access some of the earliest events of embryo development and cell fate decisions. Their potential for creating disease models, cell therapy and drug screening has generated intense interest from both scientists and the media.

ES cells have the ability to generate fully functional terminally differentiated cell types *in vitro*. The clinical relevance of this was demonstrated by the remyelination of axons by ES cell-derived ogliodendrocytes in a myelin-deficient

mouse (Liu *et al*., 2000). This research has implications in the treatment of chronic diseases such as multiple sclerosis; a progressive condition in which demyelination of axons occurs.

The propagation of ES cells is a relatively recent development (Evans and Kaufman, 1981; Martin, 1981). Understanding the molecular mechanisms that regulate fundamental ES cell biology will enable efficient culture and expansion of this cell type and uniform differentiation and tissue generation in a controlled manner.

1.1.3 Epiblast Stem Cells

Pluripotent stem cells have also been isolated from the post-implantation embryo (Brons *et al*., 2007). When the epiblast of E5.5-6.5 mouse embryos is cultured in media containing FGF2, a pluripotent population of cells termed epiblast stem cells (EpiSCs) is expanded. EpiSCs have a capacity to differentiate into many cell types *in vitro* and form teratomas exhibiting multi-lineage differentiation upon injection under the kidney capsule of severe combined immunodeficient (SCID) mice. In comparison to ES cells, EpiSCs exhibit reduced clonogenicity, a limited differentiation capacity and are unable to contribute to chimeras.

The significance of EpiSCs is their role in redefining the relationship between mouse and human ES cells (hES cells).

Despite their similar terminology, hES cells and mouse ES cells are distinct with regards to their morphology, marker expression and growth requirements. hES cells typically grow as a colony whilst mouse ES cells grow as a monolayer (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al.,* 1998). hES cells express markers SSEA3 and SSEA4 and lack SSEA1 (Thomson *et al.,* 1998) but conversely, mouse ES cells exhibit a SSEA1 positive, SSEA3 and SSEA4 negative marker profile. However, there are some similarities in marker expression, for example Oct4, Nanog and Sox2 (discussed later).

hES cells are typically cultured with a layer of irradiated embryonic fibroblasts known as feeders, which secrete a poorly defined mixture of signalling molecules sufficient to prevent differentiation (Thomson et al., 1998). Medium conditioned by these cells can also be utilised. Fibroblast growth factor 2 (FGF2) and Nodal were elucidated to be key factors in maintaining the hES cell population. (Vallier *et al*., 2005)

Xu *et al.* (2005) reported hES cells cultured in unconditioned medium showed increased BMP signalling when compared to cells cultured in feeder-conditioned medium. Differentiation was significantly repressed in the presence of FGF2 and this inhibition was enhanced with the addition of BMP antagonist Noggin. These data are supported by Dvorak *et al.* (2005), who also reported that inhibition of the FGF receptor (FGFR) causes rapid differentiation.

A study by Vallier *et al.* (2005) developed these findings, showing that TGFβ family member Nodal is necessary to maintain pluripotency. Inhibition of Activin/Nodal signalling not only induces differentiation but also blocks the action of FGF2, suggesting an essential interaction of Activin/Nodal and FGF signalling occurs.

In summary, the growth requirements of mouse and human ES cells are distinct. Mouse ES cell cultures are supported by LIF and BMP4 stimulation and hES cells require FGF2 signalling coupled with BMP antagonisation for long-term culture.

hES cells are derived from blastocyst embryos at a similar developmental stage as mouse ES cells (Thomson *et al.,* 1998), however, mouse EpiSCs are maintained by the same factors required for the maintenance of hES cells. Both mouse and human ES cells express a similar profile of core ES cell transcription factors but surface markers such as the SSEA antigens, morphology and signalling response of the cells are dissimilar. Furthermore, the pattern of gene expression of hES cells is closer to that of EpiSCs (Tesar *et al.,* 2007). This

may indicate that human ES cells represent a later stage of development than previously considered.

1.1.4 Induced Pluripotent Stem Cells

A long-term goal of many researchers is to convert somatic cells to a pluripotent state. This would permit the generation of patient-specific tissues to be used in cell therapy, drug screening and disease modelling.

Reprogramming has been achieved via the transfer of the nucleus of a somatic cell into an enucleated egg in a technique known as somatic cell nuclear transfer (SCNT) (Wilmut *et al*., 1997; Wakayama *et al*., 1997) and also through the fusion of somatic cells with pluripotent cells (Cowan *et al*., 2005; Do and Scholer, 2004). However, SCNT is inefficient and requires the use of oocytes and cell fusion produces tetraploid cells, which are unsuitable for medical applications. Despite their limitations, these techniques proved the principle that somatic cells can be converted to a pluripotent state.

Recent advances in this field showed somatic cell reprogramming is possible via direct ectopic expression of four transcription factors (Oct4, Sox2, Klf4 and c-myc) in murine fibroblasts (Takahashi and Yamanaka, 2006). These cells were termed induced pluripotent (iPS) cells.

iPS cells have the ability to form a range of differentiated tissues including the germ line (Okita *et al*., 2007) and tetraploid complementation has been achieved (Kang *et al*., 2009). The technique has been applied successfully to mouse, rat and human somatic cells and is currently being applied to an increasing number of species.

The first generation of iPS cells were isolated upon selection for Fbx15 expressing cells (Takahashi and Yamanaka, 2006). Fbx15 is a marker of ES cells. This proved an unsuitable method of selection as gene expression and DNA methylation patterns differed significantly from that of ES cells and, importantly, these cells failed to produce adult chimaeras. The utilization of

fibroblasts containing a Nanog-GFP reporter overcame these issues (Okita *et al*., 2007). Another group reported an alternative set of genes, namely Oct4, Sox2, Nanog and Lin28, also induced pluripotency in somatic cells (Yu *et al*., 2007).

One study demonstrated the medical potential of iPS cells. Human fibroblasts from five patients with idiopathic Parkinson's disease were reprogrammed and differentiated into dopaminergic neurons (Soldner *et al*., 2009). An earlier study showed neurons differentiated from rat iPS cells can integrate into the brain and improve symptoms of rats with Parkinson's disease (Wernig *et al*., 2008).

Although the potential benefits of iPS cell technology are clear, it is not yet fully understood. While progress is being made to increase efficiency, it is not clear why reprogramming is a rare event. Viral transduction of the 4 genes ensures maximum exposure of cells to the reprogramming factors, but integration of genetic material into the host cells is unsafe for medical applications. While silencing of the ectopic genes occurs upon complete reprogramming, these genes may be reactivated upon differentiation. This is supported by an observation by Okita *et al*. (2007), which described the development of tumours in mice created using iPS cells. This has been circumvented via the use of episomal vectors (Okita *et al*., 2008) or the use of recombinant proteins (Zhou *et al*., 2009), but the use of Klf4 and c-myc is unlikely to be regarded as safe for medical application due to their oncogenic nature.

ES cells remain the archetypical pluripotent cell type which iPS cells are intended to mimic. Research into both cell types will be complementary to further the understanding of these early embryonic-like cell types.

1.2 Regulation of Embryonic Stem Cells

1.2.1 Intrinsic factors

Three transcription factors play critical roles in the regulation of the transcriptional network that defines ES cell self-renewal and pluripotency; Oct4, Sox2 and Nanog.

1.2.1.1 Oct4

Oct4 is a POU class transcription factor often described as the master regulator of ES cell fate. Oct4 expression is detectable in oocytes, blastomeres, the ICM and germ line cells. The importance of this was demonstrated upon the generation of Oct4 null blastocysts. The ICM of an Oct4 deficient blastocyst develops into extraembryonic trophoblast rather than a pluripotent population (Nichols *et al*., 1998), demonstrating the importance of this transcription factor in establishing the ES cell identity. A study by Niwa *et al*. (2000) showed the level of Oct4 expression determines whether the cells adopt one of three fates. A normal level of Oct4 in the cell maintains the pluripotent ES cell population; upregulation causes endoderm and mesoderm differentiation and downregulation of Oct4 results in differentiation into trophectoderm. This suggested that transcriptional control by Oct4 is not an 'on or off' system of regulation.

Oct4 and the trophoblast regulator Cdx2 exhibit mutual repression. Cdx2 is a homeobox transcription factor and marker of trophoblast (Niwa *et al*., 2005). Oct4 represses the expression and activity of Cdx2, which in turn inhibits Oct4 auto-regulation. These data demonstrate the importance of Oct4 in establishing and maintaining a pluripotent population.

1.2.1.2 Sox2

Sex determining region Y-box 2 (Sox2) is defined as a key regulator of ES cells (Masui *et al*., 2007). Sox2 null embryos develop to stage E6.5 but derivation of

Sox2 null ES cells has not been achieved (Avilion *et al*., 2003). Conditional expression of Sox2 in ES cells results in trophoectoderm-like differentiation when Sox2 is switched off and ectopic expression of Oct4 rescues the Sox2 null phenotype (Masui *et al*., 2007). As observed with Oct4, overexpression of Sox2 does not enforce ES cell self-renewal but rather enhances neural differentiation (Zhao *et al*., 2004). Sox2 regulates the expression of transcription factors affecting Oct4 expression and with Oct4, co-ordinately regulates Oct4/Sox2 binding sites enabling regulation of target genes (Yuan *et al*., 1995).

1.2.1.3 Nanog

Nanog is a divergent homedomain transcription factor related to the Nkx family of homeodomain proteins (Chambers *et al*., 2003; Mitsui *et al*., 2003). Expression initially occurs in the morula with high levels in the early blastocyst and downregulation following implantation. In ES cells, Nanog expression oscillates in cultures maintained by Leukaemia inhibitory factor (LIF) and serum/Bone morphogenetic protein 4 (BMP4) (Chambers *et al*., 2007). This is in contrast to Oct4 and Sox2 expression, which is uniform in an undifferentiated ES cell population. It is hypothesised that this expression pattern signifies Nanog-facilitated control of the propensity of cells to undergo differentiation (Chambers *et al*., 2007; Glauche *et al*., 2010).

Deletion of Nanog results in primitive endoderm differentiation (Matsui *et al*., 2003); a fate similar to that of ES cells overexpressing GATA6. It has been suggested that Nanog may inhibit the endodermal fate via GATA6 repression. Overexpression of Nanog enables factor-independent self-renewal of ES cells (Chambers *et al*., 2003), but efficiency is further enhanced in the presence of LIF.

Approaching embryonic stage E5.5, Nanog deficient embryos degenerate into extraembryonic tissue with a loss of normal patterning. One early study reported Nanog deficient ES cells exhibited an endodermal morphology and

markers (Mitsui *et al*., 2003). These cells maintained the expression of Oct4 and Rex1 in a feeder co-culture system but this was lost without feeder support.

Another study generated an ES cell line genetically manipulated to express a tamoxifen-inducible cre recombinase and a loxP flanked Nanog transgene. These cells undergo deletion of Nanog upon addition of tamoxifen, resulting in Nanog deficient cells (Chambers *et al*., 2007). The cells were cultured extensively in the absence of Nanog and remained pluripotent with low levels of differentiation. Chimeras were successfully generated but germ cells failed to mature upon reaching the genital ridge. This suggests Nanog is dispensable for the maintenance of pluripotency but is required for the formation of germ cells.

The function of Nanog was further explored with regard to recent advances in cellular reprogramming. A somatic cell can become pluripotent upon fusion to an ES cell (Do and Scholer, 2004) and Nanog is absolutely required for the successful completion of reprogramming (Silva *et al*., 2009). Furthermore, reprogramming of neural stem cells via ectopic expression of Oct4, Klf4 and c-myc is reliant on intact Nanog expression in order to complete the transition to an ES-cell-like state.

These data imply Nanog is required for establishing rather than maintaining the state of pluripotency, and support the idea that Nanog is the ‘guardian’ of pluripotency.

1.2.1.4 Transcription Factor Network

Targets of Oct4, Sox2 and Nanog were investigated by chromatin immunoprecipitation (ChIP) analysis. A large number of targets were identified and many of these were found to interact with all three transcription factors, indicating cooperative regulation (Boyer *et al*., 2005; Loh *et al*., 2006). Genes known to be active in ES cells are regulated by these transcription factors, as are genes known to be inactive (Boyer *et al*., 2005). This suggests Oct4, Sox2 and Nanog are positive and negative regulators of gene expression. They were

also shown to promote their own expression and the expression of the other two transcription factors. Together these data imply these three transcription factors constitute a self-regulating network that establishes and maintains the pluripotent state.

1.2.2 Signals Regulating ES cell Self-Renewal

ES cells were originally derived on a fibroblast feeder layer in the presence of serum (Evans and Kaufman, 1981; Martin, 1981). It was observed that media conditioned by Buffalo rat liver (BRL) cells supported ES cell self-renewal (Smith and Hooper, 1987) and subsequent fractionation of this media revealed leukaemia inhibitory factor (LIF) as the component inhibiting differentiation (Smith *et al*., 1988; Williams *et al*., 1988).

1.2.2.1 LIF/STAT3

LIF is a protein belonging to the inter-leukin 6 (IL-6) family of cytokines. It was first described in a study demonstrating its pro-differentiative effect on M1 leukaemia cells (Tomida *et al*., 1994; Gearing *et al*., 1994). LIF binds to the LIF receptor (LIFR) and dimerises with the GP130 receptor, which increases binding affinity (Zhang *et al*., 1997).

Deletion of LIF, LIFR or GP130 does not affect normal embryonic development (Yoshida *et al*., 1996 Stewart *et al*., 1992; Ware *et al*., 1995) but GP130 is required for blastocyst development during diapause (Nichols *et al*., 1996). Downstream of the LIFR-GP130 heterodimer, Janus kinases (JAK) phosphorylate cytoplasmic domains of GP130 upon LIF induction. These phosphotyrosine residues prompt binding of SH2 domain proteins which are also phosphorylated by JAK.

The two major pathways associated with the activation of the LIFR-GP130 cascade are signal transducers and activators of transcription (STATs) (Stahl *et al*., 1995; Luttickan *et al*., 1994) and the mitogen-activated protein kinase pathway (MAPK-discussed later) (Boulton *et al*., 1994; Yin and Yang, 1994).

Phosphorylation of STAT3 by JAKs induces dimerisation and translocation to the nucleus where it functions as a transcription factor (Ihle *et al*., 1996). Activation of STAT3 signalling has been shown to activate Krüppel-like factor 4 (Klf4), which in turn promotes Sox2 (Niwa *et al*., 2009). Another downstream target of STATs is suppressor of cytokine signalling 3 (SOCS3). Induction of SOCS3 by STATs initiates a negative feedback loop wherein SOCS3 represses STAT activity (Auernhammer *et al*., 1998).

STAT3 is required for efficient self-renewal of ES cells (Niwa *et al*., 1998) and active STAT3 enables LIF-independent self-renewal in serum-based ES cell cultures (Matsuda *et al*., 1999).

In the absence of serum, LIF induces neural differentiation (Ying *et al*., 2003), indicating LIF-mediated self-renewal is serum-dependent. This implies one or more components of serum are also required for ES cell self-renewal. Ying *et al*. (2003) revealed this factor to be bone morphogenetic protein 4 (BMP4).

1.2.2.2 BMP4

BMP4 is a member of the transforming growth factor-β (TGFβ) superfamily (Shi and Massague, 2003), originally isolated from bone protein extracts and named after its ability to direct bone and cartilage formation. BMP4 signals through type I and II receptors, resulting in Smad phosphorylation. Smads are transcription factors that mediate signalling by members of the TGFβ superfamily from the extracellular environment to the nucleus.

BMP4 activates Smads1, 5 and 8, resulting in transcription of the Id genes. This may inhibit neural differentiation of ES cells through repression of mammalian achate schute homolog 1 (Mash1) (Ying *et al*., 2003). Overexpression of Id genes 1, 2 or 3 replaces the requirement for BMP4 in ES cell culture, suggesting these genes play a role in BMP4-mediated self-renewal.

BMP4 has been shown to play a role in both the maintenance of ES cell self-renewal and induction of differentiation. In serum-free culture, BMP4 is

sufficient to replace serum in supporting self-renewal (Ying *et al*., 2003). However, BMP4 induces non-neural differentiation in the absence of LIF. The identity of the large, flat, non-neural cells produced upon exposure to the BMP4 signal is currently unclear, although Harvey *et al*. (2010) recently reported the expression of early epidermal precursor markers by these cells. Interestingly, the ability of BMP4 to induce a non-neural fate is dependent on functional FGF signalling (Kunath *et al*., 2007), supporting previous studies in chick and Xenopus models (Stern, 2005).

1.2.2.3 PI3K/AKT

Insulin and insulin-like growth factors (IGFs) are present in serum (Breitman *et al.,* 1980) and at high concentration in defined media such as N2B27-based media (Cold Spring Harbor Protocols*,* 2006). The phosphoinositol-3-kinase (PI3K) pathway is a key downstream effector of these signals and is reported to play a role in both ES proliferation and self-renewal.

PI3Ks are a family of lipid kinases known to phosphorylate position 3 hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns), located at the cellular membrane.

PI3K generates phosphotidylinositol-3-phosphate (PIP3) through the phosphorylation of phosphotidylinositol-2-phosphate (PIP2) (fig1.2). PIP3 is a secondary messenger that binds to proteins bearing a pleckstrin homology (PH) domain, acting as a signal for PH domain proteins to translocate to the membrane. Two of the best-studied PH domain-containing proteins are kinases AKT and PIP3-dependent kinase 1 (PDK1).

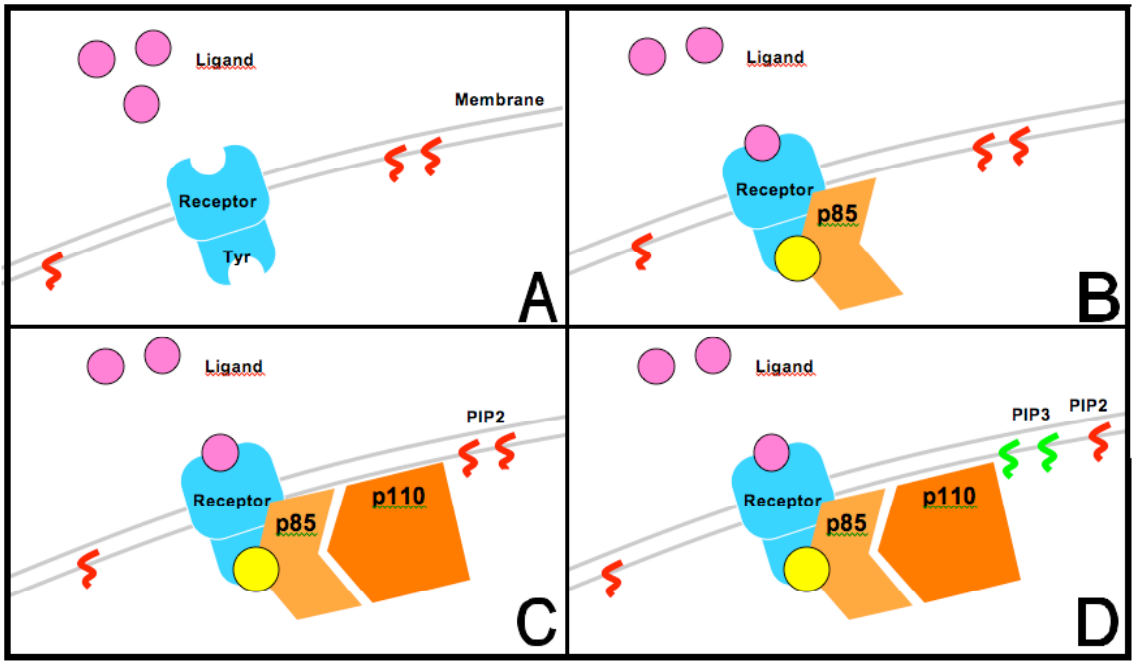


Fig1.2: Direct activation of PI3 kinase. A) Ligand is presented to receptor, B) Upon binding of the ligand, the receptor's intracellular domain autophosphorylates, presenting a phosphotyrosine residue. The regulatory subunit (here p85) of PI3K binds to the receptor as it contains SH2 domains specific to this residue (Kazlauskas, 1994; Pawson, 1995). C) The catalytic subunit (e.g. p110) is recruited to the membrane by the regulatory subunit. D) The catalytic subunit is now in close proximity to its substrate, PIP2, which is phosphorylated to form PIP3 (Reif *et al*., 1996).

PI3K activity enables translocation of AKT and PDK1 to the membrane where PDK1 phosphorylates and activates AKT (Alessi *et al*., 1997). Once activated, AKT is known to regulate multiple downstream effectors including glycogen synthase kinase β (GSK3β) (Cross *et al*., 1995), BAD (del Peso *et al*., 1997) and forkhead transcription factors (Brunet *et al*., 1999).

There are three forms of AKT; AKT1, AKT2 and AKT3. AKT1 knockout mice suffer compromised growth and a reduced lifespan without any effect on metabolism (Chen *et al*., 2001) whereas AKT2 knockout mice exhibit insulin insensitivity developing into a diabetic state (Cho *et al*., 2001). A human family presenting a similar pattern of insulin resistance and diabetes have also been diagnosed with genetically harbouring a faulty AKT2 gene (George *et al*., 2004).

AKT1 and 2 are expressed in many tissues though overexpression of one cannot compensate for the loss of another (Bae *et al*., 2003). AKT3 is predominantly expressed in nervous tissue and testes and is essential to the development of the brain rather than metabolism (Tschopp *et al*., 2005).

Inhibition of PI3K signalling occurs through dephosphorylation of PtdIns3,4,5 by phosphatase/tensin homologue (PTEN) (Maehama and Dixon, 1998) and SH2-containing inositol 5'-phosphatase-2 (SHIP2) (Backers *et al*., 2003).

PI3K plays an integral role in ES cell growth and differentiation. Inhibition of PI3K via expression of a dominant negative form of regulatory subunit p85a and also small molecule inhibitor LY294002 results in ES cell differentiation (Paling *et al*., 2004). This could be mediated by enhanced ERK phosphorylation in response to LIF or downregulation of Nanog (Storm *et al*., 2007), although MAPK activation has previously been linked to Nanog repression (Hamazaki *et al*., 2006). Watanabe *et al*., (2006) demonstrated a expression of a myristoylated form of AKT conferred constitutive activation of this pathway and enabled factor-independent ES cell self renewal.

Two signals may contribute to the constitutive activation of PI3K signalling in ES cells, T cell lymphoma protein 1 (Tcl1) and Eras. Tcl1 was discovered through global expression profiling combined with ChIP analysis of Oct4 targets (Matoba *et al*., 2006). Oct4 directly targets the Tcl1 promoter region and enhances its transcription. Overexpression of Tcl1 increases ES cell proliferation whilst suppression causes reduced proliferation. Suppression of Tcl1 is accompanied by reduced phosphorylation of AKT1 and it is suggested that this is how Tcl1 contributes to ES cell growth.

PI3K activity is also constitutively activated by the expression of an ES cell-specific homolog of Ras, Eras, which binds directly to and activates the catalytic subunit of PI3K (Takahashi *et al*., 2003). ERas exists in a constitutively active state as it is not affected by GTP-GDP exchange factors or GTPase-activating

proteins (Takahashi *et al*., 2005). It was suggested that a motif of three amino acids that is common to oncogenic forms of Ras and Eras is responsible for this lack of downregulation. These data lead to the suggestion that PI3K activity is largely regulated cell-autonomously rather than via extracellular signals.

1.2.3 Signals Regulating Differentiation

1.2.3.1 FGF4

Fibroblast growth factor 4 (FGF4) mediates its response through binding and activation of one of the four isoforms of the FGF receptor in conjunction with heparin or heparan sulphate proteoglycans (HSPG) (Rapraeger *et al*., 1991; Yayon *et al*., 1991; Ornitz *et al*., 1992). FGFs and HSPG enable dimerisation of the receptor tyrosine kinase FGFR, which autophosphorylates and recruits scaffold protein FRS (FGF receptor substrate). The active FRS presents multiple phosphotyrosine residues enabling signal amplification and activation of many downstream pathways including MAPK and PI3K (Böttcher and Niehrs, 2005)

FGF4 is critical for early development. Expression can be detected as early as the 4-cell-stage embryo (Rappolee *et al*., 1994) and FGF4 null embryos die shortly after implantation (Feldman *et al*., 1995). ES cells secrete FGF4 into the culture media and promote differentiation. FGF4 null ES cells have an attenuated differentiation capacity under defined conditions (Kunath *et al*., 2007) and this can be rescued by supplementing FGF4 to the culture media.

1.2.3.2 MAPK

Small molecule inhibition of the FGF-receptor (FGFR) by PD173074 reduces neural differentiation in mouse ES cells (Stavridis *et al*., 2007). The mitogen-activated protein kinase (MAPK) pathway was identified as the downstream effector of the FGF signal as inhibition of MAPK components has a similar effect on ES cells as FGFR inhibition. Inhibition of MAP/ERK Kinase (MEK) using PD184352 and overexpression of MAPK phosphatase 3 (MKP3), a negative

regulator of extracellular-related kinase (ERK) activity, results in a reduction in differentiation. Interestingly, these findings were transferrable to early neural specification in the chick (Stavridis *et al*., 2007).

The MAPK pathway is activated by receptor tyrosine kinases (RTKs) including FGF, LIF and Insulin/IGFs. Phosphotyrosine residues presented by activated RTKs can initiate binding of growth receptor-bound protein 2 (GRB2) (Fig1.3) and binding partner son of sevenless (SOS), a guanine exchange factor (GEF). GRB2 brings SOS into close proximity with Ras GTPase at the membrane. Ras undergoes exchange of GDP for GTP, which facilitates binding and activation of the serine/threonine kinase Raf1. This initiates a cascade of kinase activation: activated Raf1 phosphorylates MEK, which in turn phosphorylates ERK. Activated ERK dimerises and translocates to the nucleus to phosphorylate transcription factors such as Elk-1, STAT3 and myc, activating gene expression.

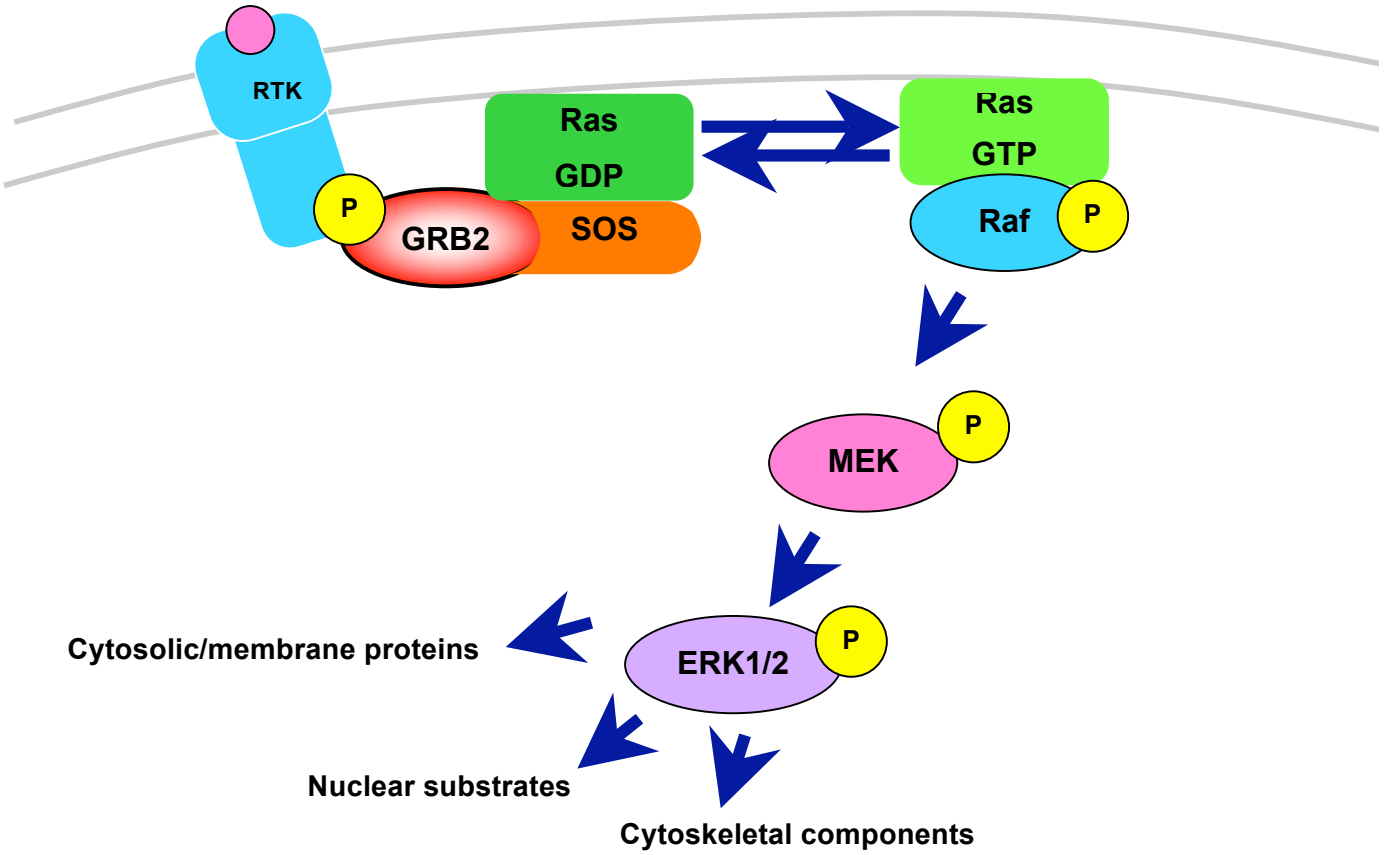


Fig1.3: The MAP-kinase pathway. Receptor tyrosine kinases or scaffold proteins present phosphotyrosine residues upon activation (Cobb *et al.,* 1989). The SH2 domain of adaptor protein GRB2 enables binding to the P-tyr (Kessels *et al.,* 2002). GRB2 also binds to son of sevenless through a SH3 domain (Simon and Schreiber, 1995). As the GRB2/SOS complex is brought to the membrane P-tyr interaction, SOS is activated and can carry out its function as a guanine exchange factor of Ras, a small GTP binding protein (Egan *et al.,* 1993). SOS exchanges GDP for GTP and Ras is able to activate Raf, a kinase of MEK (MAPK/ERK kinase), which in turn is a kinase of ERK (extracellular signal-regulated kinase). Once activated, an ERK dimer regulates cytosolic targets, cytoskeletal proteins and can also enter the nucleus to regulate various transcription factors (Khokhlatchev *et al.,* (1998).

Furthermore, while plating ES cells under MEK inhibition prevented differentiation, addition of PD184352 17-24 hours post plating failed to do so, indicating a temporal role for ERK signalling in differentiation. Stavridis *et al*. (2007) also revealed that induction of ERK activity for just one hour was sufficient to permit neural differentiation.

ERK isoforms 1 and 2 play a parallel role in FGF signalling but ERK2 is expressed more highly in ES cells. *Erk2*$^{-/-}$ embryos develop normally until after implantation when defects in mesoderm (Yao *et al*., 2003) and trophoblast (Saba-el-Leil *et al*., 2003) generation become apparent. *Erk2*$^{-/-}$ ES cells are viable, proliferate normally and express markers of pluripotency (Kunath *et al*., 2007), although their morphology is larger and flatter than wild type.

In serum-free conditions and in the absence of LIF, *Erk2*$^{-/-}$ ES cells express *Oct4, Nanog* and *Rex1*, indicating their resistance to differentiate. *Fgf5*, a marker of early differentiation, is also expressed but this is reversed upon addition of LIF, suggesting *Fgf5* expression does not signify commitment to differentiation *in vitro*. *Erk2*$^{-/-}$ ES cells form EBs poorly and fail to differentiate into mesoderm when prompted (Kunath *et al*., 2007).

Together these findings reveal ERK signalling is required for commitment to differentiation. ERK activation initiates a reversible state in which naïve ES cells become receptive to extracellular signals that promote differentiation (Fig 1.4). This naïve state has been proposed as the 'Ground State' model of ES cell identity (Ying *et al*., 2008).

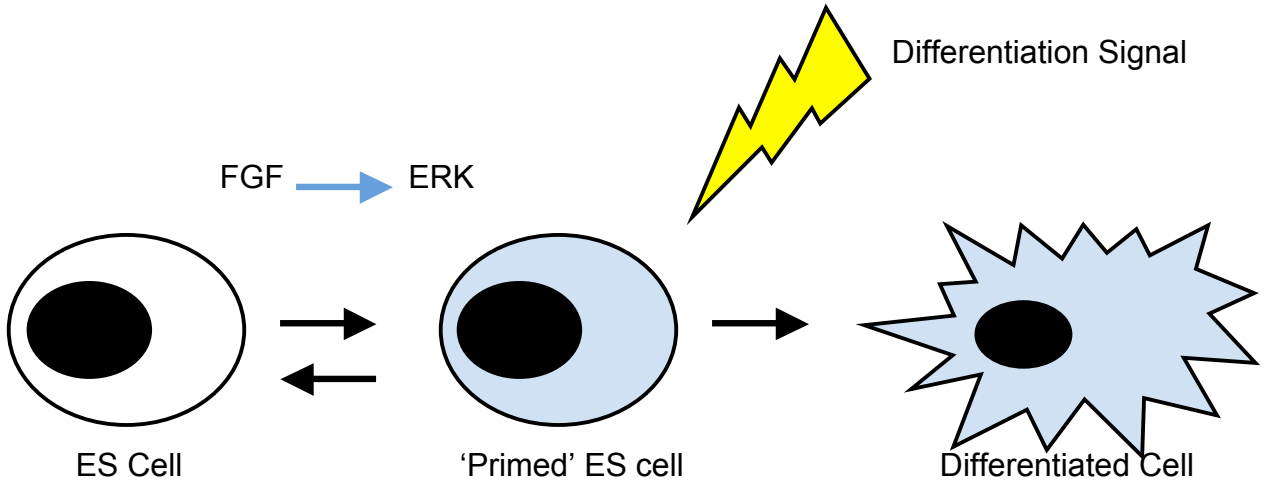


Figure 1.4. Model of ES cell commitment to differentiation. ERK activation induces a reversible 'primed' state in which ES cells are sensitive to differentiative signals (Kunath *et al*., 2007).

Serum response factor (SRF) is a cytoplasmic target of ERK known to regulate somatic proliferation in response to serum. SRF controls expression of growth promoting transcription factors such as c-fos and EGFR1. SRF null ES cells proliferate normally despite impaired serum-dependent c-fos and EGFR1 expression, indicating SRF is dispensable in ES cells (Weinhold *et al*., 2000). However, SRF null ES cells have a limited capacity to differentiate. This suggests SRF is a key downstream effector of the MAPK-mediated mechanism of differentiation.

1.3 ES cell growth

1.3.1 Cell cycle regulation

The cell cycle is a controlled sequence of events resulting in cellular division and replication. The cell cycle is composed of four phases: G1, M, G2 an S phases (Fig 1.5a) (Reviewed in Sherr, 1993). During G1 and G2 gap phases, cells increase in size. The S phase denotes a period of synthesis in which cells replicate the genome. In M (mitotic) phase, cells cease growing and undergo the controlled process of division.

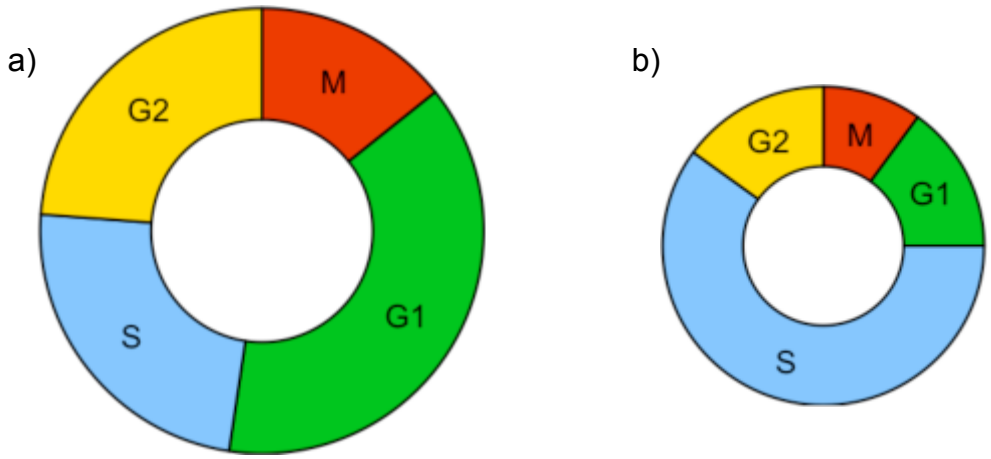


Figure 1.5 Cell cycle of a) the somatic cell and b) the ES cell. Suppression of p53 activity and the constitutive expression of cyclinE contribute to the short G1 phase exhibited by ES cells, resulting in rapid progression through the cell cycle.

The cell cycle is regulated by cyclins and cyclin-dependent kinases (CDKs) (Reviewed in Sherr, 1993 and 1994). Cyclins are non-catalytic regulatory proteins and their expression oscillates throughout the cell cycle. In contrast, CDKs are constitutively expressed binding partners of cyclins. Upon formation of a cyclin-CDK heterodimer, CDKs become catalytically active.

In the mammalian cell cycle, cyclinD binds to CDK4 and this heterodimer phosphorylates the retinoblastoma protein (Rb) (Connell-Crowley *et al.,* 1997). Activation of Rb disrupts an inhibitory complex of the E2F genes, which results

in expression of cyclinE, cyclinA and genes required for DNA synthesis (Arroyo *et al.,* 1992; Dowdy *et al.,* 1993), cyclinE binds CDK2 and this complex initiates the transition from G1 to S phase (Duronio *et al.,* 1996). A similar sequence of events occurs near the end of G2 phase, where cyclinB forms a complex with CDK1 to initiate the G2/M transition (Reviewed in Nigg, 1995).

During the somatic cell cycle, p21 induces the formation of the cyclinD-CDK4 complex (Cheng *et al*., 1999). At higher concentrations, p21 is released by the cyclinD/CDK4 complex and inhibits cyclinE/CDK2, preventing progression to S phase (Sherr & Roberts, 1999).

ES cells exhibit an unusual cell cycle in comparison to somatic cells. A rapid progression through the cell cycle is accompanied by constitutive expression of cyclin E, rather than the oscillating levels seen in somatic cells (Savatier *et al*., 1996). ES cells may lack a G1 checkpoint due to cytoplasmic sequestration of p53 (Aladjem *et al*., 1998), a checkpoint protein involved in Chk2 and p21 induction. Chk2 is a checkpoint kinase responsible for Cdc25 phosphorylation and consequent inhibition of cyclinE-CDK2 upon DNA damage while p21 is a potent cyclin-independent kinase inhibitor. p21 is undetectable in ES cells (Stead *et al*., 2002). Thus, ES cells have a short G1 phase (Fig1.5b) and express low levels of cyclinD, a regulator of the G1-S transition (Jirmanova *et al*., 2002).

In ES cells, PI3K rather than MAPK signalling regulates the transcription of cyclin D1 in contrast to somatic cells (Jirmanova *et al*., 2002). This ERK independence supports a lack of Rb control of G1 phase (Savatier *et al*., 1994).

Evidence supporting the role of PI3K in ES cell cycle regulation includes the phenotype exhibited by phosphatase and tensin homolog (PTEN) null lines. PTEN is a negative regulator of PI3K signalling and ablation of the Pten results in a 5-10% faster progression through the cell cycle (Sun *et al*., 1999). Also, deletion of Eras decreases proliferation and is restored upon expression of

constitutively active PI3K catalytic subunit p110a (Takahashi *et al*., 2003). Furthermore, inhibition of PI3K activity through the addition of small molecule inhibitor LY294002 reduces proliferation with an accumulation of cells in G1 (Sun *et al*., 1999). Downstream of PI3K, genetic ablation or rapamycin-mediated inhibition of mammalian target of rapamycin (mTOR) or results in reduced proliferation (Murakami *et al*., 2004; Takahashi *et al*., 2005).

1.4 ES Cell Differentiation

ES cells differentiate to produce cells belonging to the three germ layers known as endoderm, mesoderm and ectoderm. They can also form extraembryonic cell types although differentiation into the trophoblast lineage is rare (Dyce *et al*., 1987; Fleming *et al*., 1987).

When cultured in suspension, ES cells form aggregates termed embryoid bodies. A variety of tissues can be generated using this method. However, ES cells can differentiate in a monolayer culture. This method is thought to confer a more homogeneous environment as cells are equally exposed to extracellular signals. This tighter control over the signals received by the cells from the culture media can augment a more uniform differentiation of cells into specified lineages.

1.4.1 Endoderm

There are two major categories of endoderm; primitive and definitive endoderm. As early as stage E3.5, the ICM consists of two cell populations, identified by mutually exclusive expression of Nanog or Gata6 (Chazaud *et al*., 2006). Nanog expressing cells are fated to become the epiblast that forms foetal tissues whereas GATA6 positive cells go on to form primitive endoderm; a precursor of the extraembryonic endodermal lineage. Extraembryonic endoderm forms parietal endoderm (PE) and visceral endoderm (VE). PE migrates to form a layer inside the trophectoderm-lined cavity of the embryo and secretes extracellular matrix components of Reichert's membrane and also contributes to the parietal yolk sac. VE covers the epiblast in an epithelial layer and contributes to the visceral yolk sac. The extraembryonic lineages are vital to the survival of the developing embryo as a source of nutrition.

Extraembryonic endoderm (XEN) cell lines derived from blastocyst stage embryos proliferate indefinitely and retain their function upon injection back into the developing embryo (Kunath *et al*., 2005). ES cells have been induced to

form primitive endoderm-like cells upon withdrawal of LIF (Smith *et al*., 1988), overexpression of Oct4 (Niwa *et al*., 2000) and forced expression of Gata4 or Gata6 (Fujikura *et al*., 2002); indicating ES cells retain the ability to form extraembryonic tissue.

The definitive endodermal lineage first arises from the mesendodermal cells of the anterior primitive streak in the developing embryo. This tissue goes on to produce the gastrointestinal tract and accessory digestive and respiratory organs including lung, liver, pancreas, thyroid and thymus.

Differentiation of ES cells into endodermal lineages has been developed using increasingly specific markers. Previous methods of endoderm induction failed to consider that the position of endodermal cells in the developing embryo is a key factor in their specification. Recent studies have refined ES cell differentiation protocols to recapitulate conditions similar to those experienced *in vivo*, utilising highly specific markers to verify efficient differentiation of biologically relevant tissues (Morrison *et al*., 2008).

The anterior definitive endoderm (ADE) forms the ventral foregut and multipotent pancreatic and hepatic precursors. In this regard, understanding the mechanisms regulating efficient differentiation of ES cells into this lineage has implications for the generation of these tissues for clinical applications. ADE was formed upon exposure of an ES cell monolayer to Activin A and BMP4 for two days followed by Activin A, EGF and FGF4 for 7 days in defined media. After this time, 90% of cells were positive for Hex and Crxr4 markers, showing the majority of cells had adopted an ADE fate.

Activin A and BMP4 increased survival and proliferation of the ADE population. Inclusion of FGF4 or FGF2 increased the number of ADE cells present at day 7. It remains unclear whether FGF signalling promotes induction, survival or proliferation of ADE cells, but FGFR1 null embryos fail to produce endoderm

(Deng *et al*., 1994; Yamaguchi *et al*., 1994) therefore, this signal is critical to the endodermal specification.

1.4.2 Mesoderm

A variety of mesodermal cell types can be derived upon plating ES cells on type IV collagen in the presence of serum (Nishikawa *et al*., 1998). In order to increase the specificity of mesendodermal differentiation, Bakre *et al*. (2007) derived mesendoderm progenitor cells (MPCs). Using sustained Wnt3a or GSK3β inhibition, ES cells differentiated into a mesendodermal lineage precursor cell line which can be maintained with a stable phenotype for over one year. MPC aggregation and suspension culture results in beating cardiomyocytes in up to 70% of the aggregates, while ES cell aggregates produced beating cells in 15% of embryoid bodies (EBs). Osteogenic differentiation was also possible after 3 weeks culture in high glucose media supplemented with serum, steroid dexamethasone, vitamin C derivatives and a phosphatase inhibitor. ES cells did not survive in this media, inferring all viable cells cultured in this medium must undergo differentiation. This study suggests derivation of precursor cell lines from ES cells may increase the uniformity of directed differentiation by 'capturing' cells that are undergoing differentiation at a precursor stage.

1.4.3 Ectoderm

Neural differentiation is the best-characterised form of ectodermal specification. Two models of vertebrate neuroectodermal differentiation have been put forward. One states differentiation into the neural lineage is an instructive process, wherein Wnt and FGF are involved in the specification of the neural fate. Another model suggests neural differentiation is a default process.

Induction of neural differentiation of ES cells was originally achieved through the exposure of embryoid bodies (EBs) to serum and retinoic acid (RA) (Bain *et al*., 1995). Recently, RA was shown to drive ES cell differentiation through the initial promotion of FGF signalling followed by FGF repression (Stavridis *et al*., 2010).

Enhanced FGF signalling increases the propensity of ES cells to leave the naïve, undifferentiated state. However, upon initiation of the differentiation process, FGF signalling is prohibitive to its progression. This is demonstrated by the culture conditions defined for EpiSCs. These cells are isolated later in development compared to ES cells and require FGF signalling to promote self-renewal (Brons *et al*., 2007). RA was demonstrated to play an analogous role in chick development, suggesting this mechanism of differentiation is conserved across species.

Co-culture of ES cells with PA6 stromal cells also has neural-inducing effects (Kawasaki *et al*., 2000). It was shown that BMP antagonists cannot substitute for these feeder cells but the factor or factors contributing to differentiation remain undefined.

Ying *et al*., (2003) have established a defined, monolayer system for efficient neural differentiation. In serum-free media containing N2 and B27 supplements, ES cells undergo differentiation. After four days 60% of cells express Sex determining region Y-box 1 (*Sox1*), the earliest known neuroectodermal marker (Wood and Episkopou, 1999). After 6-8 days culture, transferring cells to fibronectin-coated plates results in GABAergic neurons. Alternatively, addition of FGF8 and Sonic Hedgehog (SHH) yields dopaminergic neurons, demonstrating the potential for the directed differentiation of neuronal subtypes. As the number of *Oct4* positive cells decreases, the number of *Sox1* expressing cells increases, but the process remains asynchronous across the ES cell population. Up to 15% of the population retains *Oct4* expression despite demonstrating full differentiation capacity upon extraction from the culture environment. Thus, the protocol does not yield a pure population of differentiated cells without sorting by FACS or drug selection.

However, the defined system described by Ying *et al*. (2003) facilitates investigation into the effect of distinct signals on neural differentiation. BMP4

inhibits neural induction in favour of undefined non-neural flat cells (Ying and Smith, 2003). However, addition of BMP antagonist Noggin does not disrupt neural differentiation in this system, despite promoting neural development in *Xenopus laevis* (Munoz-Sanjuan and Brivanlou, 2002). One explanation for this was the expression of noggin and follistatin by the cell population, which may act as autocrine inhibitors of BMP signalling.

To investigate the role of FGF signalling in neural differentiation, cells were plated at low density to reduce autocrine signalling. Addition of FGF4, but not FGF2, increased the number of *Sox1* positive, neural precursor cells by day 4 and inhibition of FGFR signalling using small molecule inhibitor SU5402 decreased this population. Also, expression of a dominant-negative FGFR reduced neural differentiation without affecting proliferation or viability, indicating a role for FGF signalling in neural differentiation.

Generation of robust and defined methods of differentiation is essential to the future of ES cells as a reliable source of tissue for clinical applications and aids the elucidation of early developmental processes. Removing the requirement for co-culture, conditioned media and cell aggregation is enabling clearer deduction of the factors required for synchronous, directed differentiation. Understanding the complex effects of autocrine signalling and direct cell-cell interactions within the cell population is one of the greatest challenges of controlled differentiation. Measures can be taken to reduce the influence of these factors, such as starting with a pure population of ground state ES cells or homogeneous precursors that are plated at low density, but it is unlikely that it will be feasible to remove them entirely.

1.5 *Grb2*

1.5.1 Structure and function

GRB2 is a small protein consisting of 217 amino acids resulting in a protein of 25kDa molecular weight. GRB2 is a ubiquitously expressed adapter protein containing two SH3 (src homology 3) domains flanking a SH2 (src homology 2) domain (Lowenstein *et al*., 1992). The SH3 domains bind poly-proline type II helices while the SH2 domain binds to phosphotyrosine motifs. This structure reflects the function of GRB2 as a non-enzymatic protein that brings together molecules bearing phosphotyrosine residues with molecules containing proline-rich motifs.

1.5.2 Upstream signals

The GRB2 SH2 domain is known to link GRB2 with many receptor tyrosine kinases, docking proteins and phosphotyrosine phosphatases bearing a pYXNX motif. A discussion of some examples follows.

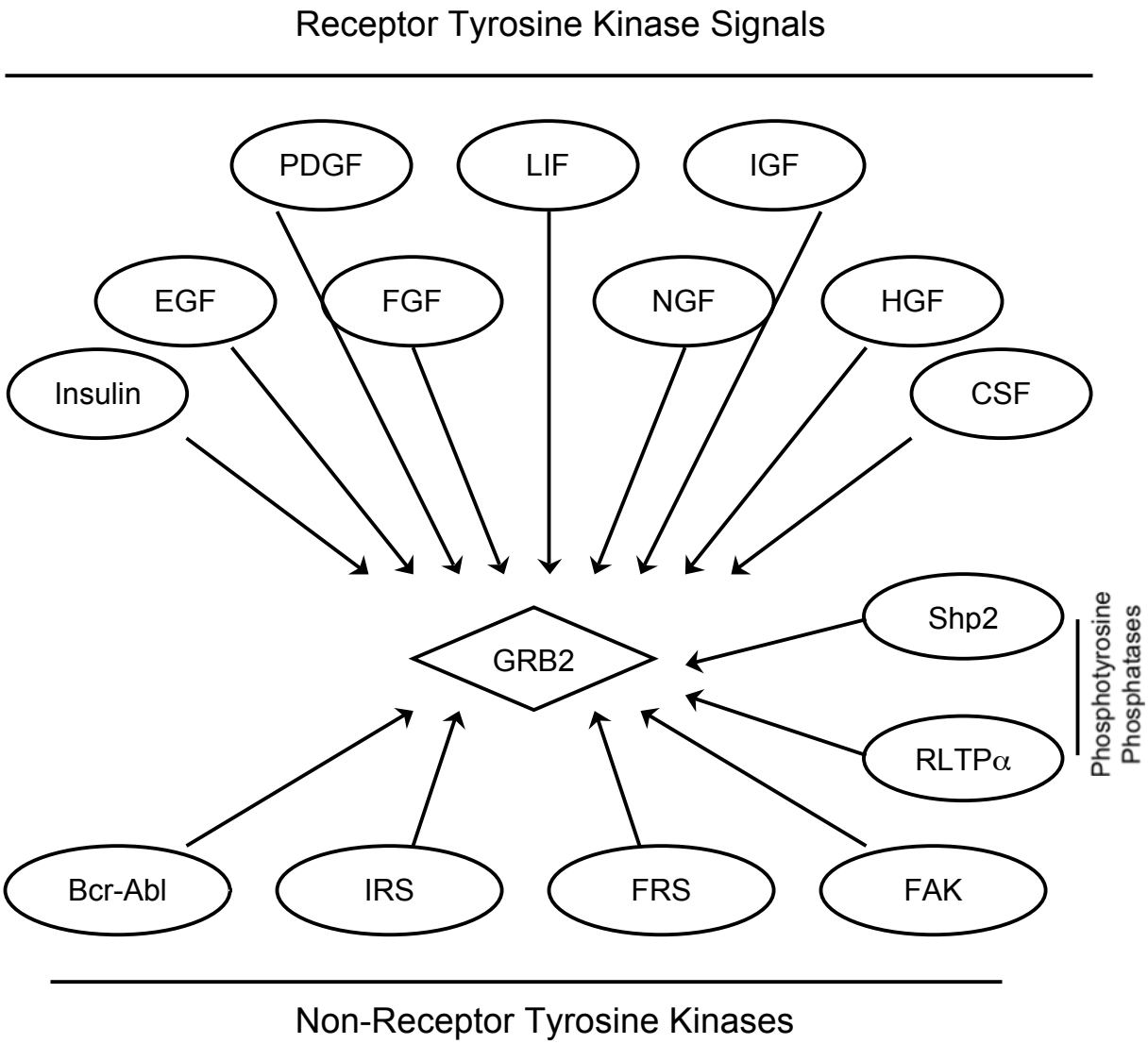


Figure 1.6 Signals upstream of GRB2.

GRB2 binds directly to phosphotyrosine residues presented by activated receptor tyrosine kinases (RTKs). Consequently, GRB2 mediates a vast number of signalling responses. GRB2 also facilitates signalling via non-receptor tyrosine kinases such as the scaffold proteins insulin receptor substrate (IRS) (Tobe *et al*., 1993) and FGF receptor substrate (FRS) (Kanai *et al*., 1997). These signalling components amplify upstream signals through the presentation of multiple phosphotyrosine residues upon recruitment to a corresponding receptor.

Phosphotyrosine phosphatase SHP2 binds directly to GRB2 via tyrosine residue 584 (Vogel and Ullrich, 1996). Shp2-mutant ES cells retain some capacity to differentiate but also maintain self-renewal upon a 4-fold reduction of LIF levels, suggesting they have increased sensitivity to the LIF signal (Qu and Feng, 1998). This is explained by the observation that Shp2-mutant ES cells exhibit increased levels of STAT3 activity in response to LIF and decreased ERK signalling (Chan *et al*., 2003), indicating SHP2 regulates both ERK and STAT3 signalling in a positive and negative fashion, respectively.

1.5.3 Downstream targets

The N-terminal SH3 domain of GRB2 binds SOS, Cbl, sprouty and dynamin via a P-x-x-P-x-R motif (Reviewed in Sparks *et al*., 1996). The C-terminal SH3 domain of *Grb2* binds to several signalling proteins containing the motif P-x-x-x-R-x-x-K-P (Lewitzky *et al*., 2001). These include GAB1 (Lock *et al*., 2000), N-WASP (Carlier *et al*., 2000), CD28 (Kim *et al*., 1998; Okkenhaug and Rottapel, 1998) and TNF-receptor I (Hildt and Oess, 1999).

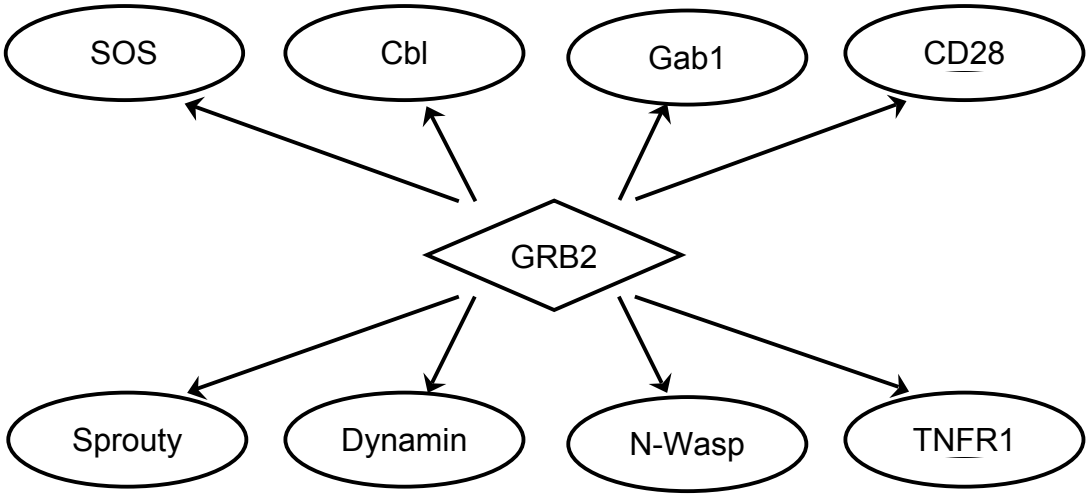


Figure 1.7 Downstream targets of GRB2

Once activated, GAB1 is phosphorylated at specific tyrosine residues and downstream effector proteins are associated via SH2 domains. Two key effectors of GAB are SHP2 and PI3K regulatory subunit p85, (discussed later).

Another downstream effector of *Grb2* is SH2–containing inositol-5-phosphatase (SHIP). SHIP is a member of the inositol polyphosphate-5-phosphatase (INPP5) family and contains an N-terminal SH2 domain, an inositol phosphatase domain, and two C-terminal protein interaction domains. SHIP removes phosphorylation from PIP3 and acts as a negative feedback mechanism for PI3K signalling.

ES cells and haematopoietic stem cells express an isoform of SHIP (s-SHIP) lacking the SH2 domain, which is found constitutively at the membrane and interacts directly with GRB2 via the SH3 domain (Tu *et al*., 2001). s-SHIP retains phosphatase activity but responds to different signals mediated by GRB2. For example, s-SHIP co-immunoprecipitates with the activated gp130 receptor (Desponts *et al*., 2006), likely through its association with GRB2. s-SHIP may also inhibit MAPK inhibition as it competes with SOS to bind to GRB2.

1.5.4 Biological Relevance

1.5.4.1 GRB2 in Cancer

Misregulation of GRB2 has been reported in many human tumours. For example, GRB2 directly interacts with Bcr-Abl, an oncogenic tyrosine kinase associated with leukemias such as chronic myeloid leukemia (Pendergast *et al*., 1993). GRB2 expression is also upregulated in breast (Verbeek *et al*., 1997), bladder (Watanabe *et al*., 2000) and prostate (Misra *et al*., 2004) cancer cell lines.

Due to its role in mediating multiple oncogenic signalling pathways, GRB2 has been designated a valuable target for anti-cancer therapy (Dharmawardana *et al*., 2006). Knockdown of GRB2 via RNA interference (Tari *et al*., 1999) or GRB2-SH2 domain-blocking peptides (Chen *et al*., 2010) have proved effective anti-cancer agents *in vitro*. Both methods report decreased proliferation of breast cancer cell lines upon downregulation of GRB2 signalling.

GRB2 has also been associated with metastasis; the process by which cancerous cells leave the primary tumour to enter the blood or lymph system and establish secondary tumours in other tissues (Steeg, 2006). Metastasis is regarded as the primary cause of death for most cancer patients and GRB2 is considered of interest as a target for inhibiting this process. Addition of GRB2 inhibitor C90 blocked Grb2/c-Met and Grb2/FAK binding, resulting in disrupted focal adhesion formation and reduced N cadherin expression and subsequently reduced solid tumour metastasis in two *in vivo* models (Giubellino *et al*., 2007).

1.5.4.2 GRB2 in Cardiac Hypertrophy

Under cardiac stress, *Grb2*$^{+/-}$ mice fail to activate p38 MAP kinase and Jun N-terminal kinase (JNK), resulting in inhibition of cardiac hypertrophy and fibrosis as observed in wild-type mice (Zhang *et al*., 2003). Mice expressing a dominant-negative form of p38α and p38β developed cardiac hypertrophy without cardiac fibrosis upon exposure to cardiac stress. This suggests a role for

Grb2 activity in cardiac hypertrophy and fibrosis and that separate signalling pathways downstream of *Grb2* regulate each of these processes.

1.5.4.3 GRB2 in Development

Grb2 is a key regulator of the MAPK pathway, which is involved in the very early stages of development. The importance of MAPK signalling to development is demonstrated in the ground state hypothesis of ES cell biology. The ground state is a hypothetical state in which an ES cell is unaffected by any process of differentiation. It is believed that ground state ES cell cultures can be achieved under defined conditions with the addition of small molecule inhibitors of GSK3β and MEK (Ying *et al.,* 2008).

Many publications have indicated that activation of the MAPK pathway causes ES cells to leave the naïve ground state and become receptive to differentiation signals (Burdon *et al.,* 1999; Kunath *et al.,* 2007; Stavridis *et al.,* 2007). As *Grb2* is a key mediator of MAPK signalling, it is possible that in its absence, ES cells have a limited capacity for differentiation due to their resistance to leave the ground state.

Deletion of *Grb2* results in embryonic lethality at approximately E4.5 when the ICM begins differentiation into endoderm in wild type embryos (Cheng *et al*., 1998).

As the formation of the ICM is not affected in *Grb2* deficient embryos, *Grb2*$^{-/-}$ ES cells can be derived. Cheng *et al*. (1998) reported *Grb2*$^{-/-}$ ES cells grow at a comparable rate to wild-type, although they show a capacity for differentiation limited to trophoblast cells. It was demonstrated that this restricted differentiation is caused by insufficient activation of SOS and therefore Ras in the MAPK pathway.

To investigate the role of *Grb2* later in development, mice were generated with one null *Grb2* allele and a hypomorphic *Grb2* allele containing a E89K substitution resulting in weakened SH2 binding (Saxton *et al*., 2001). These mice did not expire at stage E4.5 but developed abnormally with defects in many tissues including impaired survival of some branchial arch-forming neural crest cells and defective heart development. The heterozygous embryos were non-viable by E11.5 due to irregular placental tissue. Homozygous E89K *Grb2* embryos survived albeit at a reduced birth weight attributed to poor placental function.

1.6 Project Aims

Embryonic stem cells represent a transient population of pluripotent cells in the epiblast. They offer access to some of the earliest molecular events of development. Since the derivation of ES cells, great progress has been made in delineating the mechanisms of self-renewal and differentiation. However, the molecular basis of these processes is not fully understood.

Grb2 is involved in many of the key signals associated with growth and differentiation. This study aims to expand on the findings presented by Cheng *et al.* (1998) and Saxton *et al.* (2001). Both of these studies utilise serum-based media, which may mask subtle phenotypic effects of the *Grb2* deletion in ES cells. An examination of the role of *Grb2* in ES cell growth under defined conditions has not yet been described in the literature and it is important to bring previous research up to date with a more recent culture system.

The role of *Grb2* in ES cell differentiation has been described in a number of publications, but with a focus on its essential role in endodermal differentiation (Cheng *et al.* 1998; Saxton *et al.* 2001). This study aims to test the differentiation capacity of ES cells in the absence of *Grb2*, examining a wider range of lineages and differentiation markers than has previously been described.

In summary, the aims for this thesis are as follows:

- Characterise growth of *Grb2*-/- ES cells under serum-free conditions.
- Elucidate signals required for growth under serum-free conditions.
- Investigate the differentiation capacity of *Grb2*$^{-/-}$ ES cells.

The following hypotheses will be tested:

- *Grb2* signalling is required for ES cell growth under serum-free conditions.

- *Grb2* is dispensable for differentiation into some lineages.

Together, these experiments should expand on the current knowledge of signals that regulate ES cell biology.

CHAPTER 2

Materials and Methods

2.1 Cell Culture, Manipulation and Assays

2.1.1 Media, Supplements and Reagents

2.1.1.1 Media

GMEM Complete ES Cell Media

Glasgow Minimal Medium (GMEM) (Sigma, G5154)
Foetal Calf Serum (10%)
Non-essential amino acids (Gibco 11140-035)
L-Glutamine, 2mM (Gibco, 25030-024)
Sodium Pyruvate, 1mM (Gibco, 11360-039)
2-Mercaptoethanol
LIF-enriched supernatant equivalent to 100u/ml EScGro LIF (see below)

GMEM LIF-Free Media

As GMEM Complete ES Cell Media, omitting LIF.

Starvation Media

As GMEM Complete ES Cell Media, omitting LIF and serum.

N2B27 Complete ES Cell Media

1:1 DMEM:F12 (Gibco, 42430:21765)
5ml "B27+N2" supplement
6ml Non-essential amino acids (Gibco 11140-035)
500μl 2-Mercaptoethanol
LIF-enriched supernatant equivalent to 100u/ml EScGro LIF (see above)
BMP4 10ng/ml (R&D Systems, 314-BP-010)

(NB: N2 Supplement consists of: 50μg/ml BSA, 50μg/ml insulin, 6ng/ml progesterone, 16μg/ml putrescine, 30nM selenium, 100μg/ml transferrin.

"B27+N2" supplement combined these ingredients with Stem Cell Sciences B27 recipe, which is protected by CDA.)

2.1.1.2 Cytokines and Growth Factors

LIF-enriched Supernatant

Cos7 cells were transfected with a mouse LIF-encoding plasmid and supernatant was collected after 4 days. The LIF activity of the supernatant was determined by titration in comparison to EScGro (Millipore, ESG1106) recombinant LIF. ES cells were cultured for 5 days in varying amounts of LIF supernatant. Relative level of self-renewal attained was determined by staining for alkaline phosphatase activity. Quantity of supernatant used was equivalent to 100u/ml EScGro LIF.

Leukaemia Inhibitory Factor (LIF): (Millipore, ESG1107)
Bone Morphogenetic Protein 4 (BMP4): (R&D Systems, 314-BP-010)
Fibroblast Growth Factor 2 (FGF2): (Peprotech, 100-15)
Insulin-like Growth Factor (IGF1): (BA Chem, H5555)

2.1.1.3 Inhibitors and Small Molecules

ROCK inhibitor Y-27632 (Sigma, Y0503) (Li *et al.,* 2009)
GSK3β inhibitor CHIR99021 (Signalling Technologies, University of Dundee) (Ying *et al.,* 2008)
MEK inhibitor PD0325901 (Signalling Technologies, University of Dundee) (Ying *et al.,* 2008)
PKC activator Phorbol 12-myristate 13-acetate (PMA): (Sigma, P8139) (Verin *et al.,* 2000)

2.1.1.4 Other Tissue Culture Reagents

Phosphate Buffered Saline (PBS) (Oxoid, BR0014G)
Gelatin (Sigma, G1890): 0.1% in ddH_2O

Antibiotics

Puromycin (Sigma, P9620): 1.5µg/ml

Hygromycin B (Roche, 10843 555001): 200µg/ml

Cell Dissociation

Trypsin:

0.25% Trypsin (Gibco, 15090-046)

1.3mM EDTA (Sigma, E5134)

0.1% Chicken serum (Invitrogen, 16110-033)

TryplE Express (Invitrogen, 12604)

2X Freezing Mix

Serum + 20% DMSO

2.1.2 Cell lines

Grb2$^{-/-}$

R1 ES cell line carrying targeted knockout of both *Grb2* alleles (Created and used by Cheng *et al*., 1998). Kindly donated by Prof. Tony Pawson (Toronto).

CK6

Enhanced Cyan Fluorescent Protein (eCFP) expressing R1 cell line (Created and used by Hadjantonakis *et al*., 2002). Developmental potential of the cells was verified through injection into tetraploid ICR blastocysts. Obtained from Dr. Andras Nagy's lab (Toronto) (MMRRC: 011984-MU).

Fgf4$^{+/-}$

R1 ES cell line carrying targeted knockout of one *Fgf4* allele. Clone 342. (Wilder *et al*., 1997). Obtained from Dr. Angie Rizzino's lab (Nebraska).

2.1.3 Tissue Culture Common Practice

All cell lines maintained in humidified incubators at 37^{o}C, 5% CO_2

2.1.3.1 Murine Embryonic Stem Cell Culture

mES cells were routinely maintained on gelatine-coated tissue culture plastics in GMEM ES cell media. Serum-free cultures were maintained on identical gelatin-coated tissue culture plastics in N2B27 complete ES cell media. Media were changed every day and cells passaged at near-confluency.

Passage

Cells were washed once in PBS and incubated with TVP (serum cultures) or TryplE Express (serum-free cultures) for approximately 3 minutes at 37^{o}C. Detached cells were quenched using 5ml of appropriate media, pelleted by centrifugation at 1000rpm for 3 minutes and plated at the required density.

2.1.3.2 Cos7 Cell Culture

Immortalised African green monkey kidney cell line. These cells have been transformed by introduction of the SV40 genome resulting in the production of large T antigen. Cos7 cells were cultured as ES cells omitting the LIF component of GMEM media and gelatine coating.

2.1.4 Substrates

Routine culture

For routine ES cell maintenance, tissue culture plastics were coated with 0.1% gelatine at 37^{o}C for at least 10 minutes before being aspirated and media/cells added.

Substrate coatings

Laminin (Sigma, L2020)

Diluted to a final concentration of 1-2µg/cm^2 in PBS. Culture surface coated with a minimal volume and incubated at 37°C OVERNIGHT. Prior to plating cells, surfaces were washed 3 times with PBS.

Fibronectin (Calbiochem, 341668)
Diluted to a final concentration of 5µg/cm^2 in serum-free medium at 37^{o}C. Culture surface coated with a minimal volume and incubated at 37°C OVERNIGHT. Prior to plating cells, surfaces were thoroughly aspirated.

Collagen I (Sigma, C3867)
Diluted to a final concentration of 8µg/cm2 in H2O. Culture surface coated with a minimal volume and incubated at 4^{o}C OVERNIGHT. Excess fluid was removed and surfaces dried overnight. Prior to plating cells, surfaces were washed 3 times with PBS.

Foetal Calf Serum (FCS) (Biowhittaker, DE14-802F, lot 8SB013)
Culture surface coated with a minimal volume of undiluted foetal calf serum and incubated at 37°C overnight. Prior to plating cells, surfaces were washed 3 times with PBS.

Gelatin 0.1%
Diluted to a final concentration of 0.1% in H2O. Culture surface coated with a minimal volume and incubated at 37°C overnight. Prior to plating cells, surfaces were thoroughly aspirated.

2.1.5 Freezing cells

A confluent T75 flask of cells was trypsinised, pelleted and resuspended in 2ml GMEM complete ES cell media. 2ml of chilled freezing mix was added and 1 ml of this mix was aliquotted into 4 cryovials. Vials were immediately moved to -80^{o}C and transferred to -150^{o}C within 1 month.

2.1.6 Thawing cells

Cryovials were thawed at 37^{o}C. Under sterile conditions, cells were transferred to a sterile falcon with 5ml warm ES cell medium. Cells were pelleted by centrifugation for 3 minutes at 1000rpm and the supernatant aspirated. Cells were resuspended in fresh ES cell medium and transferred to a gelatinised T25 flask.

2.1.7 Genetic Manipulation

2.1.7.1 Transfection – Transient expression

Transient expression of genes was achieved through lipofection. 5x10^{5} ES cells (2.5x10^{5} cos7 cells) were plated per well of a 6-well plate overnight. Transfection mixes were prepared with a 2:5 DNA:Lipofectamine ratio. 5µl Lipofectamine 2000 (Invitrogen, 11668-019) was mixed with 125µl Opti-mem (Invitrogen, 11058-021) and incubated at RT for 5 minutes. 2µg of plasmid DNA and 125µl Opti-mem was mixed with 125µl of the diluted lipofectamine and incubated for 30 minutes at RT. After this time 250µl of the DNA/lipofectamine mix was added to the well of cells. Cells were incubated at 37^{o}C overnight (up to 16 hours) before the medium was changed.

2.1.7.2 Transfection – Stable clones

Stable integration of transgenes was achieved using the above lipofection protocol, albeit using linearised plasmid vectors. After removal of lipofectamine-containing media, cells were cultured for another 2-3 hours in fresh media and then replated into two 10cm dishes, one containing a quarter of the cells and the other containing the remaining three quarters. 24 hours after this replating, fresh media was added containing the appropriate selective antibiotic, (Puromycin – 1.5µg/ml; Hygromycin B – 200µg/ml).

Selective media was replaced every 2-3 days for up to 10 days. Colonies were ‘picked’ when large enough using a pipette tip and disaggregated in TVP for 2 minutes. The dissociated cells were replated in a well of a 24-well

plate and cultured until near confluency. These wells were expanded into a 6-well plate and frozen down upon expanding to fill the well.

2.1.8 Differentiation Protocols

2.1.8.1 Monolayer differentiation

Cells were plated at low density ($2.5x10^4$ per well 6 well plate) in LIF-free media. Cells were analysed after 5 days culture.

2.1.8.2 Embryoid Bodies

Formation of Embryoid Bodies

$2x10^6$ ES cells were plated in 10cm bacteriological (low attachment) petri dishes in 10ml of LIF-free media. Media was changed every 2-3 days. Embryoid bodies (EBs) were collected and left to settle in a 50ml falcon. Old media was aspirated and EBs resuspended in 10ml fresh media and fresh petri dishes. Care was taken not to break up or damage the EBs.

Plating EBs

After 4 or 7 days, 10-20 EBs were plated in gelatine-coated 6 well plates and media changed every 2-3 days.

2.1.8.3 Neural differentiation

Cells were cultured in N2B27 Complete ES Cell Media for one passage before being plated at low density ($5x10^3$ per well 4 well plate) in LIF and BMP4-free N2B27 media. Media were changed every 2-3 days and cells were assayed at day 7.

2.1.8.4 Laminin Differentiation

Cells were cultured in the presence of PD0325901 (1μM) and Chiron (3μM) for two passages before being plated at low density ($5x10^3$ per well 4 well plate) on laminin-coated plastics. Media were changed every 2-3 days. At day 7, cells were harvested using TryplE Express and replated at $1x10^4$ cells per well of laminin-coated 4 well plates. Cells were assayed at day 14.

2.1.9 Proliferation assays

2.1.9.1 Cell counting

Cells were plated at $8x10^5$ per well of a 6 well plate and passaged every two days. The total number of cells harvested was recorded and $8x10^5$ cells replated. This process was repeated up to 7 passages. Cumulative population doublings were calculated using the following formula: PDL = 3.32(log (total cells at harvest/total cells at seed)).

2.1.9.2 MTT assay

Kit: Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen, V13154)

The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay utilises the reduction of yellow tetrazolium MTT into a purple formazan to estimate cell numbers.

$8x10^2$ cells were plated per well 96 well plate including blank wells for background subtraction. Plates were replicated for each time point to be assayed. To assay, 1ml of MTT at 12mM in PBS was added to 10ml phenol red-free GMEM LIF-Free Media. Media was aspirated from cells to be assayed and 100µl of diluted MTT added to each well. After 1hr incubation at 37^oC the wells were lysed and solublised through the addition of 100µl 10% SDS in 0.01M HCl and incubation at 37^oC overnight. The optical densities of samples were measured at 570nm by a Multiskan Ascent plate reader (Thermo Electron Corporation).

2.1.9.3 CyQuant Direct Assay (Invitrogen, C35011)

The CyQuant Direct assay utilises a fluorescent, live cell-permeable nucleic acid dye and a suppression dye, which is impermeable to live cells. The latter dye suppresses fluorescence in all but live, intact cells. Together, these dyes label the nuclei of live cells.

$4x10^2$ cells were plated in 100µl of appropriate media per well 96 well plate including blank wells for background subtraction. Plates were replicated for each time point to be assayed. To assay the DNA content of cultures, 48µl CyQuant® Direct nucleic acid stain and 240µl CyQuant® Direct background suppressor I were mixed in 11.7ml PBS. 100µl of this combined stain was added on top of cells in culture and incubated at 37°C for 1hr. Fluorescence intensity was measured using a 488/520nm filter on a Victor^2 1420 Multilabel Counter (Wallac).

2.1.10 Adhesion assay

$0.8x10^6$ cells were plated per well of a gelatine-coated 6 well plate. At set time points media was removed, cells carefully washed once with PBS and the remaining, adherent cells were harvested and counted.

2.1.11 Annexin V: FITC Apoptosis Detection Kit II (BD Biosciences, 556570)

At the onset of apoptosis, phosphatidyl serine residues are exposed on the outer leaflet of the cell. This change can be detected using Annexin V (Vermes *et al*., 1995). Annexin V is a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidyl serines. Conjugated with a fluorochrome such as FITC, it can be used to label cells with exposed phosphatidyl serines. Loss of membrane integrity is a later event in apoptosis and necrosis (Kerr *et al*., 1972) and can be measured using a live cell-impermeable nucleic acid stain such as propidium iodide stain.

Reagents

10X binding buffer (0.1M Hepes/NaOH (pH 7.4), 1.4M NaCl, 25mM $CaCl_2$) diluted 1:10 with dH2O

FITC conjugated Annexin V

Propidium iodide

Triplicate wells were passaged three times in N2B27 Complete ES Cell Media with parallel GMEM Complete ES Cell Media cultures. Cells were carefully harvested and $4x10^6$ cells resuspended in 1ml X1 Binding Buffer. 100µl samples were taken and cells were incubated with/without 10µl FITC-conjugated annexin V solution at RT for 15 minutes in the dark. After this incubation, 300µl 1X Binding Buffer was added to each sample. 5µl propidium iodide solution was added immediately before analysis by flow cytometry.

2.1.12 PI cell cycle analysis

Fixation

Cells were harvested to a single cell suspension. $1x10^6$ cells were resuspended in 50µl PBS and fixed in 1ml ice cold 80% ethanol. Samples were stored at 4°C for at least 24 hours prior to staining

Staining

Samples were pelleted, ethanol aspirated and washed in 1ml PBS.

Staining solution

69µM propidium iodide
500µg/ml RNase A
38µM trisodium citrate solution
0.5% NP-40

Washed cells pelleted again and resuspended in 500µl staining solution. Samples incubate at RT in dark for 15 minutes. Samples vortexed immediately prior to flow cytometry analysis.

2.1.13 Staining protocols

2.1.13.1 Leukocyte Alkaline Phosphatase Kit (Sigma, 86R)

Alkaline phosphatase is a hydrolytic enzyme and a marker of pluripotent ES cells. Although other somatic cell types also express alkaline phopshatase, loss of alkaline phosphatase activity is associated with loss of pluripotency during early differentiation. The kit identifies cells exhibiting alkaline phosphatase activity through incubation in a mixture of naphthol AS-BI alkaline solution with fast red violet LB. The resulting insoluble diffuse, red dye deposit indicates sites of alkaline phosphatase activity.

Fixation

25ml Citrate solution (18mM Citric acid, 9mM Na citrate, 12mM NaCl)

8ml Formaldehyde

65ml Acetone

Media was removed from cells in culture. Cells were washed once with PBS and fixed for 45 seconds. Fixed cells were washed with ddH_2O.

Staining

0.2ml FRV alkaline solution

0.2ml Na Nitrite solution

9ml ddH2O

0.2ml Napthol solution

FRV and Na Nitrite solutions were mixed and incubated at RT for 2 minutes. ddH_2O was added and then Napthol solution. Complete stain was kept in dark. Fixed cells were aspirated and incubated with stain for 15 minutes at RT in the dark. Stain was rinsed with two ddH_2O washes and brightfield microscopic images taken of plates using a Scion CFW-1608C camera.

2.1.13.2 Immunohistochemistry

PBST: 1XPBS + 0.3% Triton X-100
Blocking solution: 10% goat serum + 1% BSA in PBST

Cells were washed once with PBS and fixed with 4% PFA for 10 minutes. Fixed cells were washed twice with PBS briefly and four times with PBST for 5 minutes. Cells were incubated for 2 hours in blocking solution and probed overnight at 4^{o}C with primary antibodies diluted in fresh blocking solution. See table 2.1 for antibody dilutions.

Cells were washed four times with PBST for at least 5 minutes and incubated for 2 hours at RT in the dark with appropriate secondary antibodies diluted 1:1000 in fresh blocking solution. Cells were washed four times with PBST for at least 5 minutes and stained with 1ng/ml DAPI (Invitrogen, D1306) in PBS for 10 minutes at RT in dark. DAPI was rinsed with PBS and stained cells were stored in dark at 4^{o}C before images were taken.

Table 2.1 Antibodies

Antigen	Source	Class	Company	Cat No	Dilution
βactin	Mouse	IgG1	Santa Cruz	sc-47778	1:2000
pERK1/2	Rabbit	IgG	Cell Signaling	9101	1:1000
ERK2	Mouse	IgG	BD Transduction	610104	1:2000
pAKT	Rabbit	IgG	R&D Systems	AF887	1:1000
AKT	Rabbit	IgG	Cell Signaling	9272	1:1000
GRB2	Rabbit	IgG	Santa Cruz	sc-255	1:2000
pGSK3β	Rabbit	IgG	Cell Signaling	9336	1:1000
GSK3β	Rabbit	IgG	Cell Signaling	9315	1:1000
pSTAT3	Rabbit	IgG	Cell Signaling	9131	1:1000
STAT3	Rabbit	IgG	Santa Cruz	sc-482	1:1000
Oct4	Mouse	IgG2b	Santa Cruz	sc-5279	1:200
β Tubullin III	Mouse	IgG2a	Covance	MMS-435P	1:200
Nestin	Mouse	IgG1	DSHB	Rat401	1:20
Nanog	Rabbit	IgG1	Abcam	ab80892	1:200
Anti Rabbit IgG HRP	Goat	IgG	Cell Signaling	7074	1:5000
Anti Mouse IgG HRP	Goat	IgG	Cell Signaling	7076	1:5000
AlexaFluor Secondary Antibodies	Goat	Various	Invitrogen		1:1000

2.2 Molecular Biology

2.2.1.1 Biochemical induction

Cells were plated $1x10^6$ per well of a 6 well plate and incubated overnight at 37^oC. Cells were washed once with PBS and incubated in 1ml starvation media for 3hrs. If an inhibitor was used, cells were treated for one hour after starvation. Cells were induced with chemical or cytokine and immediately harvested for protein.

2.2.1.2 Preparation of Protein Lysates

1XSDS buffer:

10% Glycerol
3%SDS
62.5mM Tris HCl pH6.8
0.005% Bromophenol Blue
3% 2-Mercaptoethanol

Cells were washed once with PBS and immediately placed on ice. 100µl 1X SDS buffer was used to cover entire surface and lysates were scraped and transferred to 1.5ml eppendorf tube. Samples were sonicated for 8 minutes in an XL2020 Ultra Sonic Processor (Misonix Inc) and stored at -20^oC.

2.2.1.3 Western Blotting

Table 2.2 Buffers and Reagents

10% Resolving gel
6ml d H_2O
5ml 40% acrylamide - Fisher #BP14021
3.2ml 2% Bis solution - BioRad #161-0142
5ml 1.5M tris pH 8.8
200µl 10% SDS
200µl 10% Ammonium persulphate
12µl Temed - Sigma #T9281-50ml

10% Stacking gel
3.3ml d H_2O
625µl 40% acrylamide
400µl 2% Bis solution
650µl 1M Tris pH 6.8
50µl 10% SDS
50µl 10% Ammonium persulphate
5µl Temed

5X Running buffer
30g Tris
188g glycine
10g SDS
upto 2L H_2O

1X Running buffer
200ml 5X Running buffer
upto 1L d H_2O

10X Transfer buffer
29.3g Glycine
58g Tris
18.8ml 20% SDS
upto 1L H_2O

1X Transfer buffer
100ml 10X Transfer buffer
200ml ethanol
upto 1L d H_2O

Block
5% Marvel milk in TBST

Primary antibody dilutant
5% BSA (Sigma, A9647) in TBST

25X TBS
125g Tris
400g NaCl
upto 2L H_2O
pH7.6

1X TBST (Wash buffer)
80ml TBS
2ml Tween 20 (Sigma, P7949)
upto 2L d H_2O

Secondary antibody dilutant
10% Blocking Solution in TBST

2.2.1.3.1 SDS PAGE Gel Preparation

The resolving gel was mixed and poured immediately into cassette (Biorad, 345-9903) and sealed with a layer of isopropanol. Once set, the isopropanol was removed and stacking gel was mixed and poured on top. A comb was inserted and the gel was left to set. Wells were syringed prior to loading samples.

2.2.1.3.2 Sample Preparation

Prior to loading, samples were thawed at 37^{o}C and boiled at 95^{o}C for 5 minutes.

2.2.1.3.3 Loading/Running gel

8µl of sample or molecular weight marker was loaded per well. Empty wells were filled with 1X SDS buffer. Gels were ran at 50V until molecular weight markers began to resolve at which point the voltage was increased to 100V. The gel finished running when the bromophenol blue band ran off the bottom.

2.2.1.3.4 Wet Transfer

Membrane soaked in dH_2O on rocker 10 minutes, then transferred to chilled 1X transfer buffer for 10 minutes. Gel and membrane were sandwiched in a transfer cassette between two fibre pads and two pieces of chromatography paper. Once inserted into transfer tank in the correct orientation, the tank was filled with chilled 1X transfer buffer, placed in a bucket of ice and ran at a constant voltage of 95V for 75 minutes.

2.2.1.3.5 Blocking

The membrane was washed in PBS for 2 minutes and transferred to blocking solution for 1hr at RT or overnight at 4^{o}C.

2.2.1.3.6 Primary antibody

The membrane was rinsed in TBST to removed excess block. Primary antibody was diluted as described in table 2.1 and membrane probed for 1hr or overnight.

2.2.1.3.7 Primary wash

Membrane was washed briefly three times and for 15 minutes three times in TBST.

2.2.1.3.8 Secondary antibody

Appropriate, HRP-conjugated secondary antibody diluted 1:5000 and membrane probed for 1hr

2.2.1.3.9 Secondary Wash

Membrane washed briefly three times and for 15 minutes three times in TBST.

2.2.1.3.10 Development

Membrane was treated with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, RPN2109) for 1min and signal captured using Amersham Hyperfilm (GE Healthcare, 28-9068) using exposures ranging from 10 seconds to overnight.

2.2.1.3.11 Stripping

Membrane was rinsed in TBST for 5 minutes and stripped with Restore stripping buffer (Thermo Scientific, 21059) for 15 minutes at RT. Membrane was rinsed twice in TBST for 5 minutes and blocked for 1hr before next probe.

2.2.2 RNA Methods

2.2.2.1 RNA Extraction

RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, 74104) and QIAshredder Homogenization columns (Qiagen, 79654) following manufacturer's instructions. Samples were eluted in RNAse-free water.

2.2.2.2 Quantification

RNA was quantified using a Nanodrop 1000 and accompanying software (Thermo Scientific).

2.2.2.3 cDNA synthesis from RNA

cDNA was synthesised from RNA using Superscript First Strand Synthesis System (Invitrogen, 11904-018) using oligo-dT primers following manufacturer's instructions.

RNA	– 8µl (5ng-5µg)
10mM dNTP mix	– 1µl
0.5µg/µl Oligo(dT)12-18 primer	– 1µl

The above mix was incubated at 65^{o}C 5 minutes then on ice 1min.

In a new tube (per reaction):

(in order)

10X RT buffer	– 2µl
25mM MgCl2	– 4µl
0.14M DTT	– 2µl
RNaseOUT	– 1µl

9µl of above reaction mix was added to the RNA:primer mix, mixed gently and incubated at 42^{o}C 2 minutes. 1µl SS II RT (or 1µl DEPC-treated H_2O for no RT control) was added to each tube and incubated at 42^{o}C 50 minutes then the reaction was heat killed at 70^{o}C 15 minutes. Reactions were sat on ice, 1µl RNase H added and incubated at 37^{o}C 20 minutes. cDNA samples were stored at -20^{o}C.

2.2.3 DNA methods

2.2.3.1 Polymerase Chain Reaction (PCR)

PCR Reaction Mix

Reagents were mixed on ice (in order):

upto 50µl	Nuclease-free H_2O
5µl	X10 buffer (Invitrogen, 10342-020)
1.5µl	50mM MgCl2
0.8µl	25mM dNTPs
1µl	10µM Oligo Fwd
1µl	10µM Oligo Rev
2µl	cDNA (200ng/µl)
0.5µl	Taq polymerase (Invitrogen, 10342-020)

Samples were amplified using the DNA Engine Dyad Peltier Thermal Cycler running the following program:

94°C 2min

94°C 30s
50°C 40s
72°C 40s
} 25-30 cycles

72°C 10min

Reactions were run on a 2% agarose TBE gel to check for correct size of amplicon.

2.2.3.2 Primers

Table 2.3 Primer Sequences

mRNA	*Direction*	*Sequence*
Gata6	For	CCCACTTCTGTGTTCCCAATTG
	Rev	TTGGTCACGTGGTACAGGCG
Gata4	For	TCCTGTGCCAACTGCCAGAC
	Rev	TGGTGGCGTTGCTGGAGTTG
Sox17	For	AGGAGAGGTGGTGGCGAGTAG
	Rev	GTTGGGATGGTCCTGCATGTG
Brachyury	For	GTGACTGCCTACCAGAATGA
	Rev	ATTGTCCGCATAGGTTGGAG
Snail	For	GTCTGCACGACCTGTGGAA
	Rev	CAGGAGAATGGCTTCTCACC
Twist	For	AGCTACGCCTTCTCCGTCT
	Rev	TCCTTCTCTGGAAACAATGACA
Nestin	For	AGAGAAGCGCTGGAACAGAG
	Rev	AGGTGTCTGCAACCGAGAGT
Sox1	For	AGATGCACAACTCGGAGATC
	Rev	CTTCTTGAGCAGCGTCTTGG
Pax6	For	GAGACTGGCTCCATCAGACC
	Rev	CTAGCCAGGTTGCGAAGAAC
Map2	For	TTAAACAGGCGAAGGATAAAGTCAC
	Rev	TGATTGCAGTTGATCCAGGGGTAG
Cdx2	For	GCGACAAGGGCTTGTTTAGA
	Rev	GAGGGAAGGGACAGGAAGTC
Oct4	For	TTCAGAGGTGCTGGGGATC
	Rev	CAATGCTAGTGATCTGCTGC
Nanog	For	GCCCTGAGAAGAAAGAAGAG
	Rev	CTGACTGCCCCATACTGGAA
Fgf5	For	ACCCTTTGAGCTTTCTACCC
	Rev	CCGTCTGTGGTTTCTGTTGAGG
Grb2	For	TACGAGGAAACTGGACGTTC
	Rev	TTAAAGATCAGTGTTTAAAT
B-actin	For	GGCCCAGAGCAAGAGAGGTATCC
	Rev	ACGCACGATTTCCCTCTCAGC

2.2.3.3 Restriction digest

DNA was digested using enzymes and appropriate buffers (New England Biolabs/Roche) for one hour (analytical) or two hours (preparative).

2.2.3.4 Agarose gel electrophoresis

Analytical gels were prepared and run using 1X TBE buffer (Ambion, AM9865) whilst preparative gels were made and run with TAE (40mM Tris-acetate, 2mM EDTA). Buffers were mixed with the appropriated amount of agarose (Invitrogen, 15510-027) and heated in a microwave until the agarose was completely dissolved. Once set in a casting tray, gels were transferred to an electrophoresis tank containing buffer, loaded and ran at 50V to resolve bands.

2.2.3.5 DNA visualisation

Gels were bathed in dilute ethidium bromide for 10 minutes prior to visualisation. Images of gels were taken using a Gel Logic 200 system (Kodak). Preparative gels were visualised using a shortwave UV transilluminator to minimise UV exposure.

2.2.3.6 Gel extraction

DNA bands were excised from preparative gels using a clean scalpel blade and extracted using the Gene Clean II (MP Biomedicals, 1001-400) according to manufacturer's instructions. DNA was eluted in ddH_2O. Water was chosen to elute the DNA as it does not contain salts that may affect applications such as ligation and sequencing.

2.2.3.7 Chloroform extraction

100µl chloroform was added to 100µl DNA and vortexed. The solution was spun at 13000rpm in a standard benchtop micro centrifuge for 2 minutes. The DNA-containing supernatant was transferred to a fresh Eppendorf tube and the protocol repeated. This procedure was immediately followed by an ethanol precipitation.

2.2.3.8 Ethanol precipitation

200µl absolute ethanol and 10µl Na Acetate pH5.2 was added to 100µl DNA and mixed by inversion. The stringy DNA precipitate was pelleted at 13.2rpm in a standard benchtop micro centrifuge for 10 minutes. After two washes in 80% ethanol the pellet was air dried for 10 minutes and resuspended in an appropriate volume of RNase-free H_2O.

2.2.3.9 Quantification

DNA was quantified using a Nanodrop 1000 and accompanying software (Thermo Scientific).

2.2.3.10 Ligation

Ligation reactions were set up using 400units T4 DNA ligase (NEB, M0202), following manufacturer's instructions. A molar ratio of 1:3, insert:vector DNA was used with 100ng vector DNA. Ligation mixes, including a vector only control, were incubated at 16^{o}C overnight.

2.2.3.11 Transformation

Competent cells, Subcloning Efficiency DH5α Competent Cells (Invitrogen, 18265-017), were thawed on ice. 50µl of cell suspension was mixed with 5µl DNA, including a pUC19 plasmid positive control, and incubated on ice for 30 minutes. Cells and DNA were heat shocked at 42^{o}C for 45 seconds and then incubated on ice for another 2 minutes. 950µl warm SOB (Super Optimal Broth, 2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 8.56mM NaCl, 2.5mM KCl, 10mM MgCl2 in ddH_2O) was added and cells incubated at 37^{o}C, 225rpm for 1hr. The cells were spun briefly in a benchtop centrifuge and excess supernatant poured off. Cells were resuspended in residual media and spread onto warm LB agar plates (1.5% agar in LB (Lysogeny Broth, 1.0% Tryptone, 0.5% Yeast Extract, 1.0% Sodium Chloride)) containing 100ug/ml Ampicillin (Sigma, A9393). Plates were incubated at 37^{o}C overnight.

2.2.3.12 Plasmid DNA propagation and purification

2.2.3.12.1 DNA Miniprep

Miniprep cultures were cultivated from one colony picked into 4ml LB plus 100µg/ml ampicillin (Sigma, A9393) and incubated at 37^{o}C, 225rpm overnight. Cultures were spun down and DNA purified using Wizard Plus SV Miniprep kit (Promega, A1460), following manufacturer's instructions. DNA was eluted in nuclease-free H_2O.

2.2.3.12.2 DNA Maxiprep

Maxiprep cultures were cultivated from one colony picked into 250ml LB plus 100µg/ml ampicillin and incubated at 37^{o}C, 225rpm overnight. Cultures were spun down and DNA purified using Purelink HiPure Plasmid Filter Maxiprep kit (Invitrogen, K2100-17), following manufacturer's instructions. DNA was eluted in nuclease-free H_2O.

CHAPTER 3

Grb2-/- ES cells Do Not Grow Efficiently in N2B27-LB Medium.

3.1 Introduction

3.1.1 Defined/Undefined Culture Conditions and their Effects on ES cell Identity

Embryonic stem cells are derived from the inner cell mass of the blastocyst embryo (Evans and Kaufman, 1981). They represent a population of cells in the embryo that go on to form the tissues of the foetus. ES cells are defined by two characteristics; the capacity to expand in culture indefinitely and the ability to differentiate into the three germ layers of the developing embryo, a property known as pluripotency.

Three transcription factors play a critical role in the regulation of ES cells; *Oct4*, *Sox2* and *Nanog*. *Oct4* and *Sox2* exhibit uniform expression across a self-renewing ES cell population (Scholer *et al*., 1989; Yuan *et al*., 1995). However, the level of Nanog expression oscillates in each cell (Chambers *et al*., 2007). Two additional ES cell markers; *Rex1* and *Stella*, also exhibit variable levels of expression across the population (Toyooka *et al*., 2008; Hayashi *et al*., 2008). *Rex1* or *Stella* negative ES cells can resume expression of *Rex1* or *Stella* without becoming fully committed to differentiation. These data demonstrate the inherent heterogeneity of ES cell populations.

Since their first derivation, significant progress has been made in understanding and controlling the identity of the ES cell through modifications to their culture conditions.

ES cells were originally derived, expanded and maintained in serum-based media in co-culture with murine embryonic fibroblasts (MEFs) (Evans and

Kaufman, 1981). The mechanism by which MEFs and serum supported ES cell self-renewal and proliferation was unclear. Smith *et al*. (1988) reported the first feeder-free ES cell cultures. These were established using a defined substrate coating, gelatin, and LIF.

Further research identified targets of LIF activation. LIF induces activation of two major pathways; *Stat3* and MAPK. *Stat3* is a key signal for the promotion of ES cell self-renewal (Niwa *et al*., 1998). In contrast, MAPK signalling via LIF is dispensable for this process (Burdon *et al*., 1999).

For many years, LIF and serum-based media have been used for the propagation of ES cells. Serum is a complex media component derived from the non-cellular fraction of coagulated blood. The use of serum is regarded as a source of complexity and inconsistency in ES cell culture. Recent advances identified BMP4 as a suitable substitute for serum (Ying *et al*. 2003). BMP4 was shown to promote the transcription of Inhibitor of differentiation (Id) genes.

Utilising specific factors in place of serum reduces the potential of exposing ES cells to pro-differentiative signals that may promote heterogeneity within the ES cell population. Using a combination of LIF, BMP4 and N2 and B27 supplements, Ying *et al*. (2003) established a fully defined, robust system for the propagation of ES cells.

Interestingly, the mechanism by which LIF and BMP4 support the culture of ES cells is to block differentiation rather than promote self-renewal. In the absence of BMP4, LIF is incapable of blocking neural differentiation (Ying *et al*., 2003) whilst BMP4 promotes non-neural differentiation (Ying *et al*., 2003; Zhang *et al*., 2010). Together, LIF and BMP4 inhibit the progression of differentiation into all lineages, resulting in the maintenance of self-renewal.

Nonetheless, even under these fully defined conditions, the ES cell population remains heterogeneous. This may be due to the effect of autocrine signalling via FGF4. FGF4 signalling is known to inhibit *Nanog*

expression (Hamazaki *et al*., 2006) and promote differentiation (Kunath *et al*., 2007; Stavridis *et al*., 2007).

These data culminated in a new strategy for the expansion and maintenance of the ES cell population. Ying *et al*. (2008) utilised small molecule inhibitors of specific signals in order to prevent differentiation of ES cells. Addition of inhibitors of the FGF receptor (SU5402) and MEK (PD184352), a component of the FGF-MAPK pathway, to N2B27-based media resulted in the expansion of undifferentiated cells for multiple passages. This suggests inhibition of pro-differentiative signalling by FGF and MAPK is sufficient to promote self-renewal. However, cells failed to thrive under these conditions, particularly at low density. This was overcome by the addition of an inhibitor of GSK3β (CHIR99021). When combined with FGF receptor and MEK inhibition, this inhibitor mimicked the effects of both AKT and Wnt signalling to promote ES cell growth.

ES cells under FGF receptor inhibition by SU5402 exhibit homogeneous expression of Nanog (Hamazaki *et al*., 2006). This supports *in vivo* data demonstrating that FGF receptor and MEK inhibition results in homogeneous expression of Nanog throughout the blastocyst-stage epiblast (Nichols *et al*., 2009). It is reported that inhibition of FGF signalling increases the homogeneity of an ES cell population (Lanner *et al*., 2010). The identity of the cells under 3i culture has been described as ‘naïve’. This suggests the cells are in a state of self-replication that is unaffected by any process associated with the progression of differentiation. This homogeneous status reported in ES cells and their *in vivo* equivalents has been termed the ‘Ground State’ (Ying *et al*., 2008).

3.1.2 ES Cell Growth

Self-renewal encompasses both the inhibition of differentiation and the promotion of ES cell proliferation. The Ground State hypothesis claims ES cells employ an intrinsic mechanism of growth wherein extracellular signals are not required (Ying *et al*., 2008).

PI3K is a major pathway involved in the regulation of growth in many cell types. Two signals may contribute to the constitutive activation of PI3K signalling in ES cells, T cell lymphoma protein 1 (Tcl1) and *Eras*. *Tcl1* was discovered through global expression profiling combined with ChIP analysis of *Oct4* targets (Matoba *et al*., 2006). *Oct4* directly targets the *Tcl1* promoter region and enhances its transcription. Overexpression of *Tcl1* increases ES cell proliferation whilst suppression causes reduced proliferation. Suppression of *Tcl1* is accompanied by reduced phosphorylation of AKT1 and it is suggested that this is how *Tcl1* contributes to ES cell growth.

PI3K activity is also constitutively activated by the expression of an ES cell-specific homolog of *Ras*, termed *Eras*, which binds directly to and activates the catalytic subunit of PI3K (Takahashi *et al*., 2003). Eras exists in a constitutively active state as it is not affected by GTP-GDP exchange factors or GTPase-activating proteins (Takahashi *et al*., 2005). It was suggested that a motif of three amino acids that is common to oncogenic forms of *Ras* and *Eras* is responsible for this lack of downregulation. These data lead to the suggestion that PI3K activity in ES cells is largely regulated cell-autonomously rather than via extracellular signals.

3.1.3 *Grb2*

GRB2 consists of a SH2 binding domain flanked by two SH3 domains. The SH2 domain associates with phosphotyrosines presented by activated receptor tyrosine kinases (RTKs) and docking proteins such as the insulin receptor substrate (IRS) and the FGF receptor substrate FRS. The two key pathways mediated by GRB2 are MAPK and PI3K/AKT.

Upon binding to a phosphotyrosine residue, GRB2 binds to a SH3 domain-containing protein, Son of sevenless (SOS), recruiting it to the membrane. Here SOS is activated and carries out its function as a guanine exchange factor of *Ras*, a small GTP binding protein. SOS exchanges GDP for GTP, thus activating *Ras*. Active *Ras* induces a cascade of phosphorylation in the MAPK pathway, including Raf, MEK and ERK kinases.

GRB2 can also activate PI3K/AKT through the recruitment of SH3 domain-containing GRB2-associated binding protein (*Gab*). This docking protein directly recruits PI3K to the membrane resulting in the activation of the PI3K pathway.

3.1.4 *Grb2* Regulates Growth

Grb2 has been implicated in the regulation of growth in various systems. *Grb2* is directly recruited by activated of Vascular endothelial growth factor receptor-3 (VEGFR3) in Human Umbilical Vein Endothelial Cells (HUVECs) (Salameh *et al*., 2005) resulting in the promotion of proliferation through activation of ERK1/2 and AKT signalling pathways. *Grb2* also promotes proliferation via heregulin-ErbB2 signalling in human breast cancer cell lines (Tari *et al*., 1999; Lim *et al*., 2000). AKT, rather than MAPK/ERK was shown to be the key downstream signal in these cell types.

A study into cultured smooth muscle cells derived from *Grb2*+/- and *Grb2*+/+ mice showed *Grb2*+/- cells were resistant to neointima development in response to vascular injury (Zhang *et al*., 2003). Neointima is a term used to describe new vascular tissue generated in response to injury or the presence of aprosthesis. Generation of this tissue requires the migration and proliferation of smooth muscle cells. The role of *Grb2* in proliferation of aortic smooth muscle was confirmed upon knockdown of the *Grb2* mRNA transcript. *Grb2* was targeted through the introduction of morpholino oligonucleotides into primary rat aortic smooth muscle cells (RAOSMCs). A reduction in GRB2 protein levels was observed, with reduced proliferation and MAPK signalling.

3.1.5 The *Grb2*$^{-/-}$ ES Cell Phenotype

Deletion of *Grb2* results in embryonic lethality at approximately E4.5 when the ICM begins differentiation into endoderm in wild type embryos (Cheng *et al*., 1998). From these embryos, a *Grb2* deficient (*Grb2*$^{-/-}$) ES cell line was derived. While *Grb2*$^{-/-}$ ES cells grow at a rate comparable to wild type, they exhibit a limited capacity for differentiation.

As the formation of the ICM is not affected in *Grb2* deficient embryos, a

Grb2$^{-/-}$ ES cell line was derived. Cheng *et al*. (1998) reported *Grb2*$^{-/-}$ ES cells grow at a comparable rate to wild type cells and exhibit a restricted capacity for differentiation which is limited to trophoblast cells. It was demonstrated that this restricted differentiation is caused by insufficient activation of SOS and therefore *Ras* in the MAPK pathway.

These findings were expanded upon with the report that the ICM of *Grb2*$^{-/-}$ embryos consist of a population of cells exhibiting uniform expression of Nanog as opposed to a mixture of Gata6 and Nanog positive cells (Chazaud *et al*., 2006). *Grb2*$^{-/-}$ ES cells are also resistant to sodium vanadate-mediated Nanog repression (Hamazaki *et al*., 2006).

3.1.6 Aim

No defect in proliferation is observed in *Grb2*$^{-/-}$ ES cells (Cheng *et al*., 1998). However, the behaviour of *Grb2*$^{-/-}$ ES cells in defined media has not been reported. The aim of this chapter is to investigate the role of *Grb2* in ES cell growth under fully defined conditions.

3.2 Results

3.2.1 *Grb2* ES Cells Grow Poorly in N2B27

To begin investigating the requirement of *Grb2* in ES cells, wild type and *Grb2*$^{-/-}$ ES cells were cultured in serum-based GMEM Complete ES Cell Medium (GMEM+FCS) and serum-free N2B27 Complete ES Cell Medium (N2B27-LB). No difference was observed upon plating wild type or *Grb2*$^{-/-}$ lines in GMEM+FCS or N2B27-LB during the first passage. However, differences between the lines emerged at the second passage in N2B27-LB. In N2B27-LB, wild type ES cells appeared normal (fig 3.1). In contrast, *Grb2*$^{-/-}$ cells grew in tight, refractive colonies with indistinguishable cell boundaries in this media.

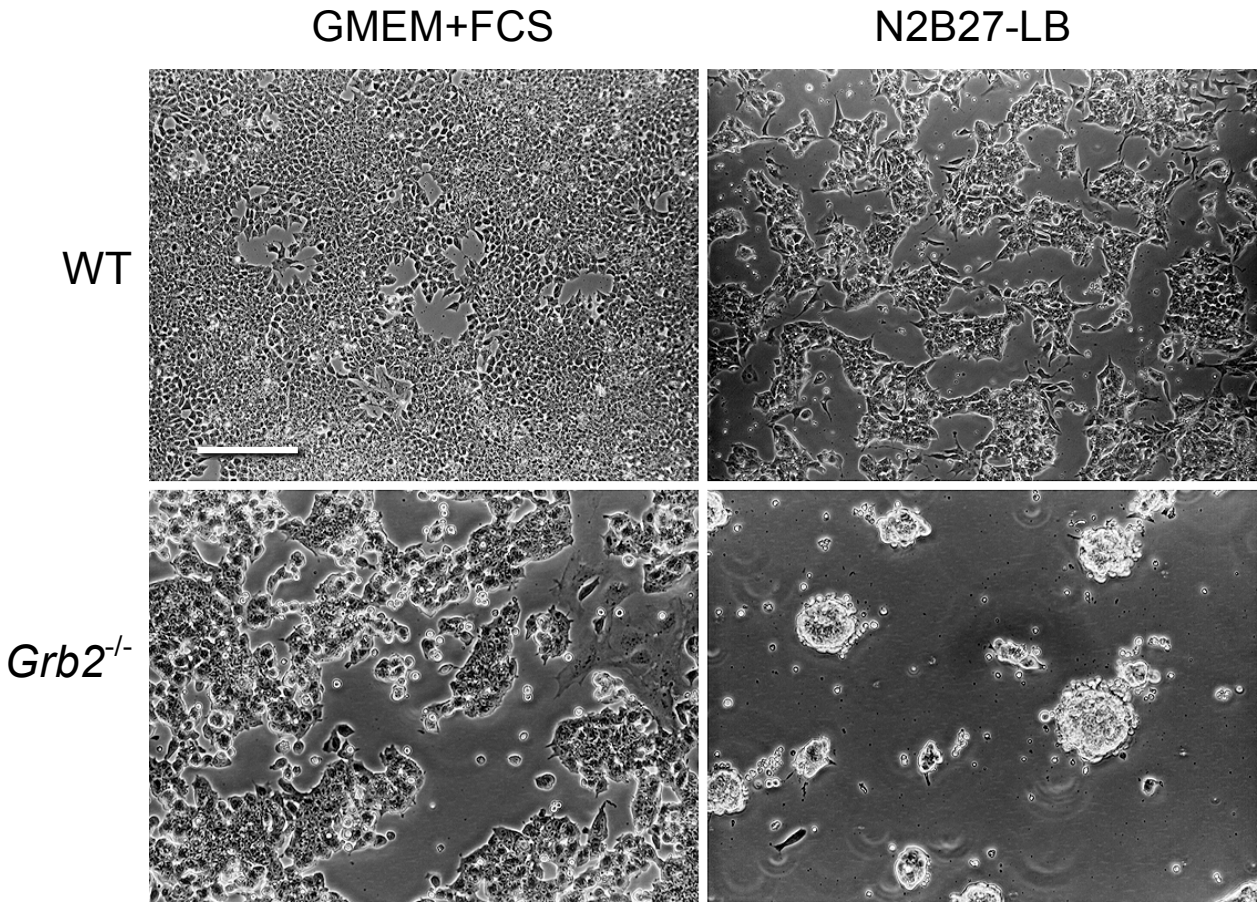


Figure 3.1 *Grb2*$^{-/-}$ ES cell exhibit an unusual morphology when cultured in N2B27-LB. Cells grow in tight, refractive colonies in which individual cells are difficult to distinguish. Scale bar – 100μm.

N2B27-based media were originally developed in order to cultivate neuronal cells (Bottenstein *et al*., 1979; Brewer *et al*., 1993) and upon withdrawal of LIF and BMP4, ES cells can differentiate into neural lineages (Ying *et al*., 2003). Although *Grb2*$^{-/-}$ ES cells are reputed to be unable to differentiate, their behaviour under defined conditions is yet to be reported. It was, therefore, important to investigate whether cells retained expression of stem cell markers. The status of the cells was confirmed through immunohistochemical staining for two key ES cell markers; transcription factors *Oct4* and Nanog.

These proteins were located in the nuclei of the majority of cells (fig3.2), consistent with established expression pattern of ES cells. In GMEM+FCS medium, wild type and *Grb2*$^{-/-}$ cells exhibited differing levels of Nanog expression across the ES cell population. However, *Grb2*$^{-/-}$ ES cells stained very highly and uniformly for Nanog in N2B27-LB, whilst wild type ES cells retained variable levels.

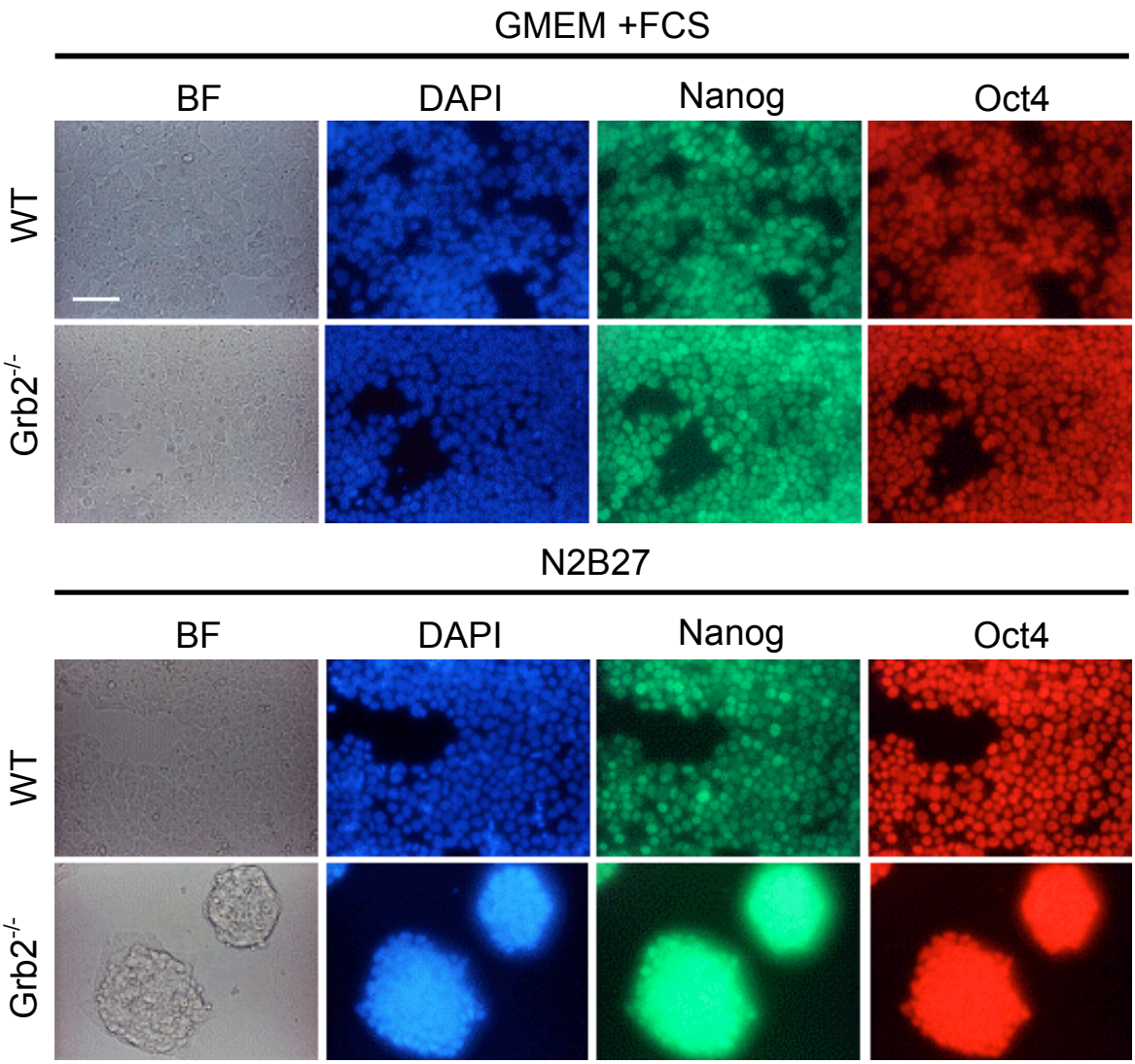


Figure 3.2 *Grb2*$^{-/-}$ ES cells stain intensely for ES cell markers *Oct4* and *Nanog*. Scale bar – 25μm.

The change in morphology of *Grb2*$^{-/-}$ cells switched to N2B27-LB suggested that there might be a defect in growth related to *Grb2* deficiency in this medium. To establish whether there was a defect in growth, a cell counting assay was performed. 0.8×10^6 wild type or *Grb2*$^{-/-}$ ES cells were plated per 6 well plate in GMEM+FCS or N2B27-LB media and cultured for 2 days. After 2 days, the number of cells was recorded and re-plated at the same density in fresh 6 well plates. The population doublings were calculated and graphed cumulatively. Figure 3.3 shows no significant difference between wild type

and *Grb2*$^{-/-}$ ES cells in GMEM+FCS and significantly ($p<0.001$ by student's T-test) diminished population doublings by *Grb2*$^{-/-}$ ES cells in N2B27-LB medium compared to wild type.

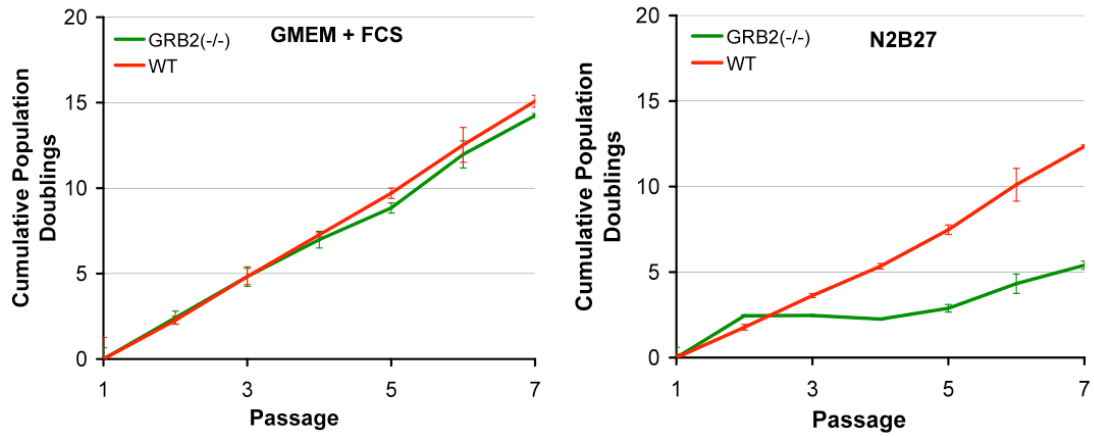


Figure 3.3 *Grb2*$^{-/-}$ ES cell exhibit a reduced growth rate in N2B27-LB medium. $0.8x10^6$ wild type and *Grb2*$^{-/-}$ cells were plated per well of a 6 well plate. After 2 days culture, cells were counted and replated to $0.8x10^6$/well. The number of cumulative population doublings was calculated and graphed (n=3 from 3 experiments).

To support this result using an alternative, quantitative method, an MTT assay was performed. The MTT assay is a colourmetric assay that measures cell numbers based on the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole substrate to purple formazan by live, metabolically active cells. $8x10^2$ wild type or *Grb2*$^{-/-}$ ES cells were plated per well of a 96 well plate in GMEM+FCS or N2B27-LB. Replicate plates were made for each assay timepoint. Cells were assayed at 4, 24, 48 and 72 hours. Data was normalised to the 4-hour timepoint to account for potential differences in the number of cells plated in the assay. Figure 3.4 shows no significant difference in cell numbers was measured between wild type and *Grb2*$^{-/-}$ ES cells cultured in GMEM+FCS ($p=0.12$ by student's T-test). However, in N2B27-LB medium, *Grb2*$^{-/-}$ cells exhibited reduced cell numbers in comparison to wild type ($p=0.012$ by student's T-test). These data suggest that in the absence of *Grb2*, ES cells fail to proliferate efficiently.

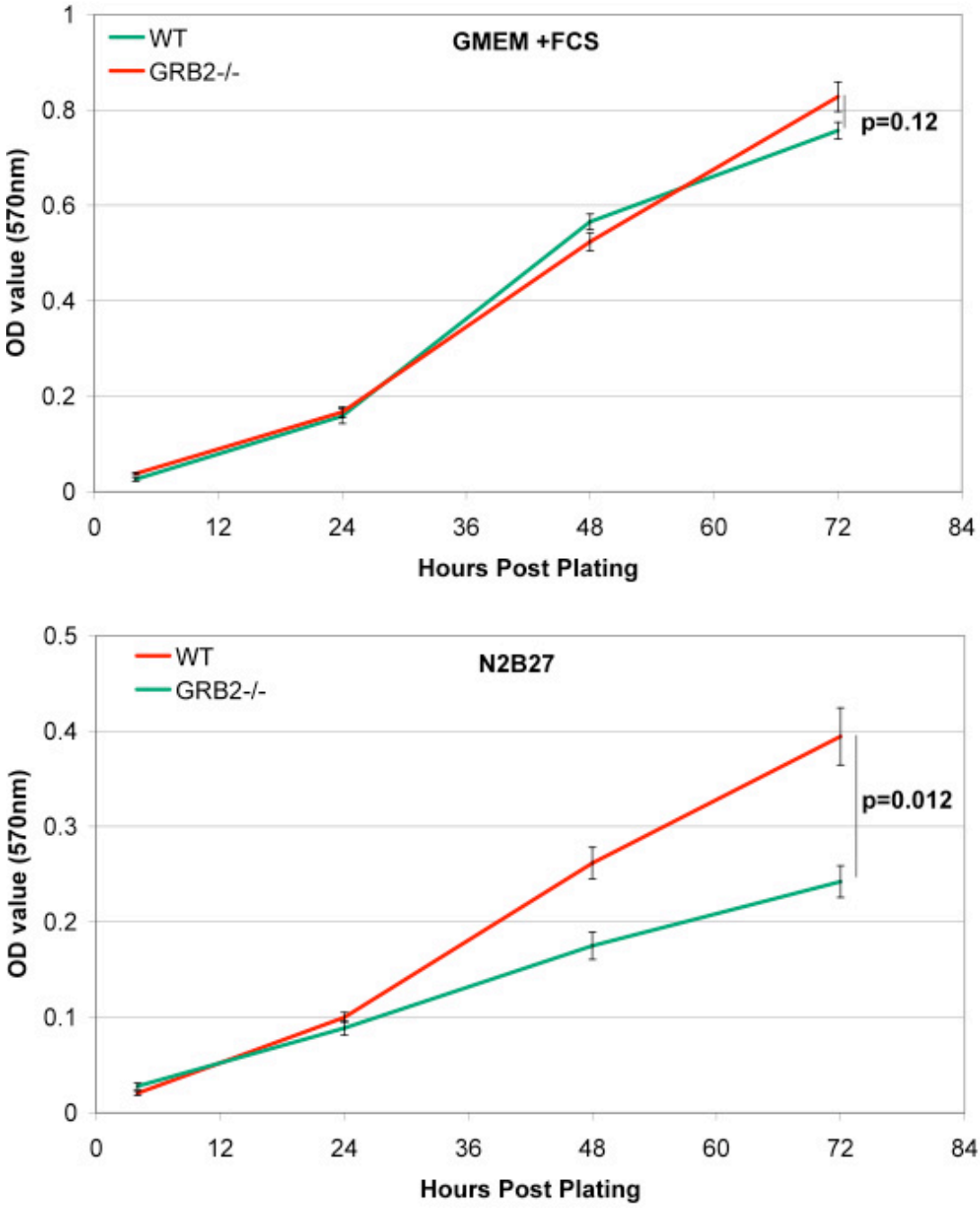


Figure 3.4 *Grb2*$^{-/-}$ ES cell exhibit a reduced growth rate in N2B27-LB medium. 8x10^2 wild type or *Grb2*$^{-/-}$ cells were plated per well of a 96 well plate. Replicate plates were created for each time point. At 4, 24, 48 and 72 hours, cells were incubated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 1 hour before overnight solublisation. The conversion of yellow MTT to purple formazan was quantified using a spectrophotometric plate reader with a 570nm filter. Data was normalised to the 4-hour time point and a student's T-test performed on the endpoint dataset (n=12 from 2 experiments).

3.2.2 Restoration of *Grb2* Expression Restores Normal Growth.

The *Grb2*$^{-/-}$ cells were generated through the genetic manipulation of an R1 ES cell line (Cheng *et al*., 1998). Through homologous recombination, a 6kb deletion of exons 2 and 3 from the *Grb2* gene resulted in truncation of the *Grb2* locus. The remaining exons 1 and 4 were frameshifted, ensuring no expression of *Grb2* was possible. Selection for successful deletion of *Grb2* was based on resistance to G418. In order to select for cells in which both *Grb2* alleles have been targeted, Cheng *et al*. (1998) subjected the cells to high levels of G418 and clonal selection.

To exclude the possibility that the poor growth phenotype exhibited by *Grb2*$^{-/-}$ ES cells was due to clonal variation, *Grb2* expression was restored in *Grb2*$^{-/-}$ ES cells. This was achieved through the construction of a *Grb2*-expressing vector.

Grb2 cDNA was excised from the plasmid vector by NotI/SalI restriction digest and ligated into a NotI/XhoI digested pCAGS-IRES-PURO^{R} vector. Both plasmids are derivatives of the pPyCAGIP vector published by Chambers *et al*. (2003) (fig 3.5a).

The constructed plasmid contains various elements that confer its function (fig 3.5b). In order to confirm its functionality, this vector and a complementary empty vector acting as a negative control were expressed transiently in a cos7 cell line. This immortalised monkey kidney cell line expresses the SV40 large T antigen, which is defective in genomic DNA replication (Gluzman, 1981). As the *Grb2* construct contained the SV40 promoter, it would be substantially amplified if the vector was correctly assembled. This would result in the production of a large amount of protein, which would enable easy detection by Western blot.

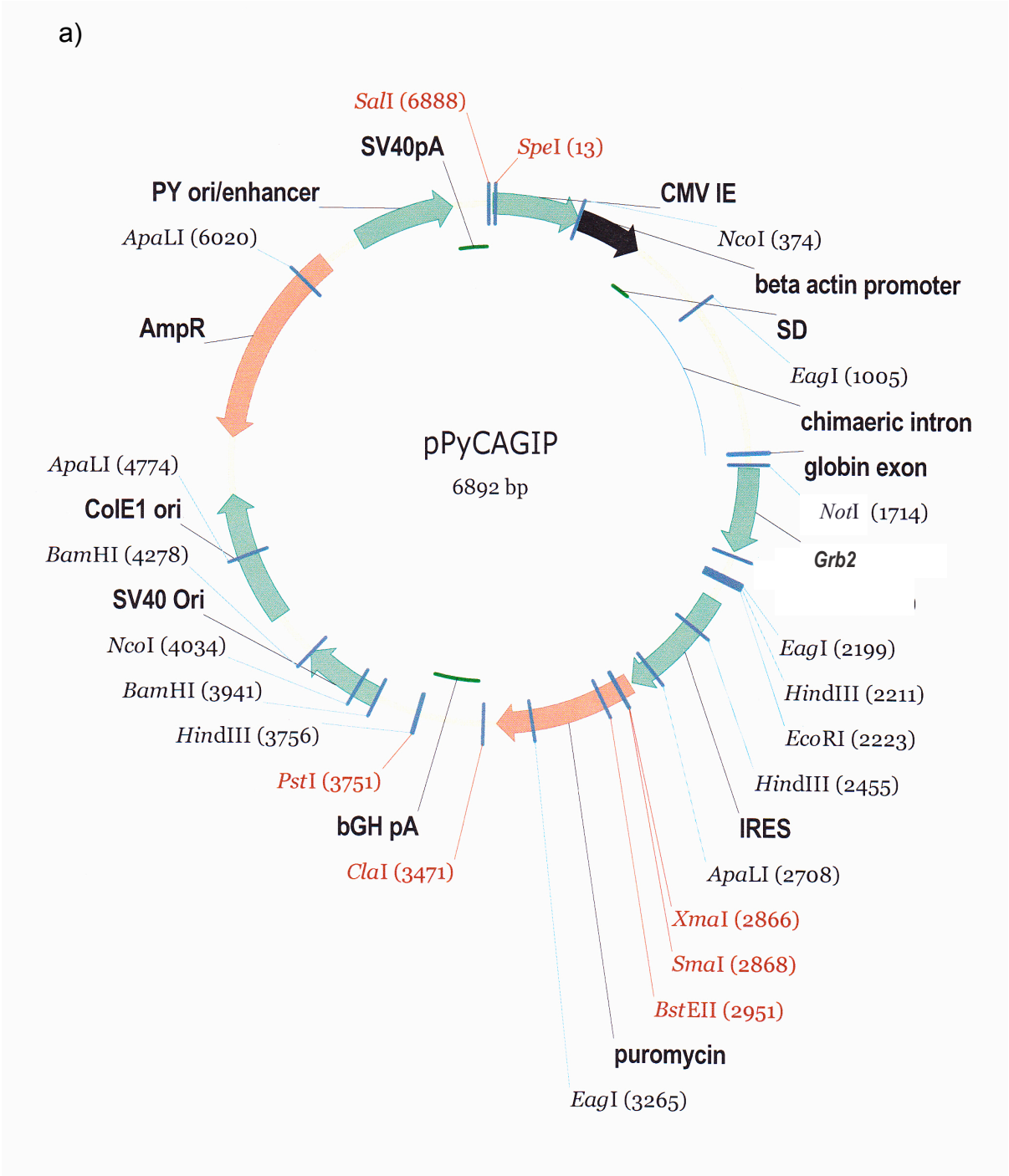


Figure 3.5 a) Restriction map of the CAGS-*Grb2*-IRES-IP.

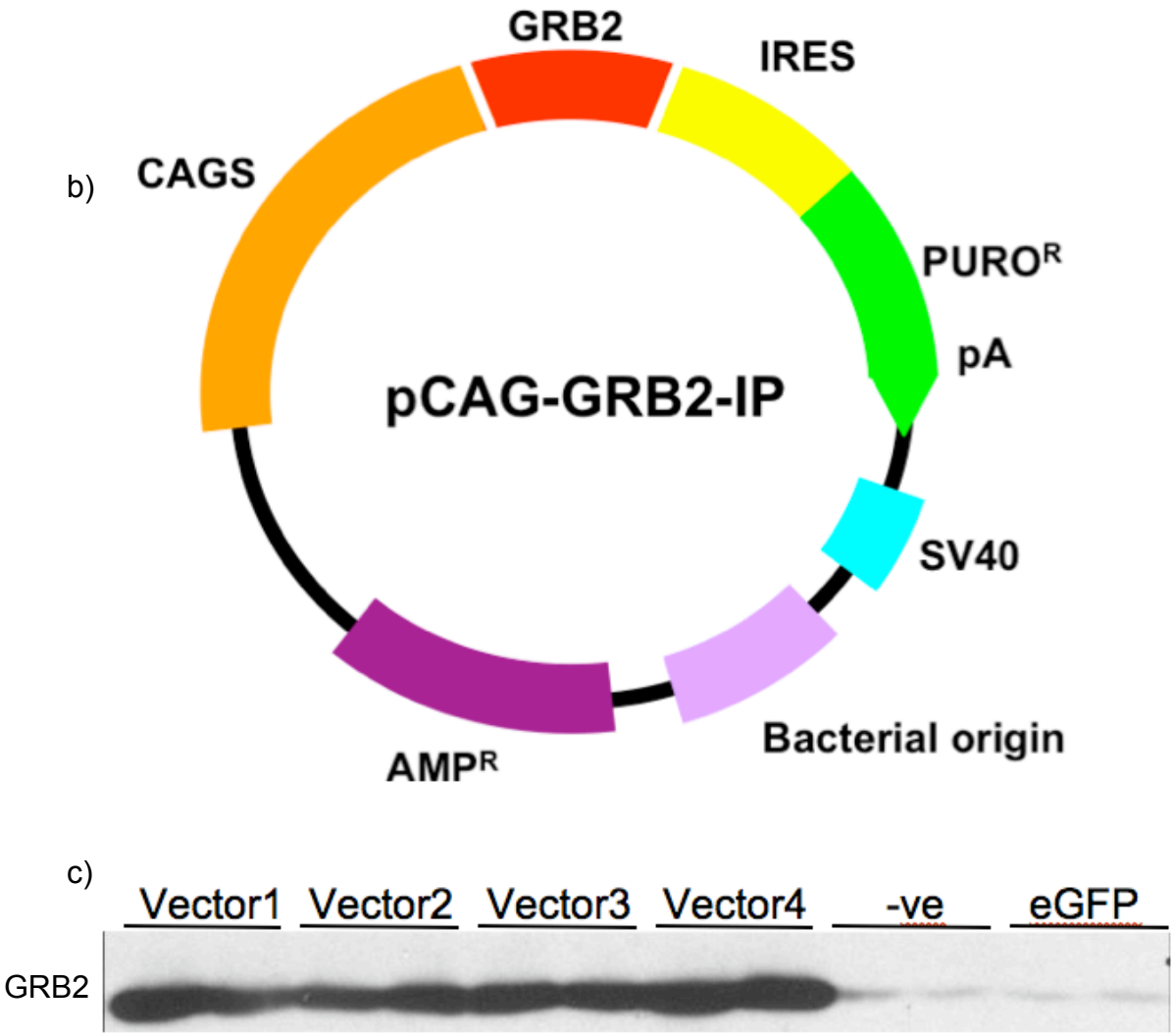


Figure 3.5 b) Schematic of the CAGS-*Grb2*-IRES-IP expression vector **CAG** - The CAG promoter encompasses the cytomegalovirus (CMV) early enhancer element and chicken β-actin promoter (Alexopoulou *et al.,* 2008). This combination results in high levels of gene expression and is especially effective in ES cells, which are known to silence the CMV promoter alone (Xia *et al.,* 2007). **IRES** - The internal ribosome entry site (IRES) element enables initiation of translation from within a mRNA molecule (Pelletier and Sonenberg, 1988). Linking a gene of interest to the sequence encoding a selection marker via an IRES element improves the likelihood that the level of gene expression is comparable to selection marker expression. **PURO^R** - Puromycin N-acetyletransferase (PAC) is an enzyme produced by *Streptomyces alboniger* that acetylates and inactivates puromycin, imparting resistance to this antibiotic (Gómez Lahoz *et al.,* 1991). **pA** - Addition of multiple adenosine nucleotides to the tail of an mRNA increases that stability of the transcribed mRNA and enhances translation (Guhaniyogi and Brewer, 2001). **SV40** - The SV40 promoter interacts with cells expressing the SV40 large T antigen, which is defective in genomic DNA replication, resulting in increased vector amplification (Gluzman *et al.,* 1981). **Bacterial Origin/Amp^R** - These elements enable amplification of the vector upon transformation into bacterial cells.

c) Transiently transfected Cos7 cells express GRB2 protein. Four *Grb2* expression vectors, an empty CAGS-IRES-IP vector and eGFP-expressing vector were lipofected into Cos7 cells. Western analysis of protein lysates shows high levels of GRB2 protein expression in *Grb2*-transfected cells. Cell transfected with the empty vector or eGFP-expressing vector exhibit low, endogenous levels of GRB2 protein expression.

To test if the *Grb2* expression cassette was functional the *Grb2* vectors were transiently transfected into Cos7 cells and cell lysates were analysed by western blotting. A low level of endogenous GRB2 (25 kDa) was detectable in lysates transfected with eGFP-expressing and empty vectors. Figure 3.5c shows cells transfected with any of the four *Grb2*-containing vectors expressed very high levels of GRB2. This confirms that all four vectors are functional.

One of the verified constructs was transfected into *Grb2*$^{-/-}$ ES cells and after 10 days in puromycin-containing, selective media, stable clones were picked and expanded. The remaining clones were fixed and stained for alkaline phosphatase (AP) activity and counterstained with Leishman's stain to visualise the non-AP positive cells. These stains revealed differences between *Grb2* and empty vector transfected colonies (Fig3.6). Empty vector (*Grb2*$^{-/-}$) transfected colonies appeared highly AP positive whereas *Grb2* transfectants typically exhibited a large area of flat, AP negative cells with differentiated morphology, surrounding an AP positive central core.

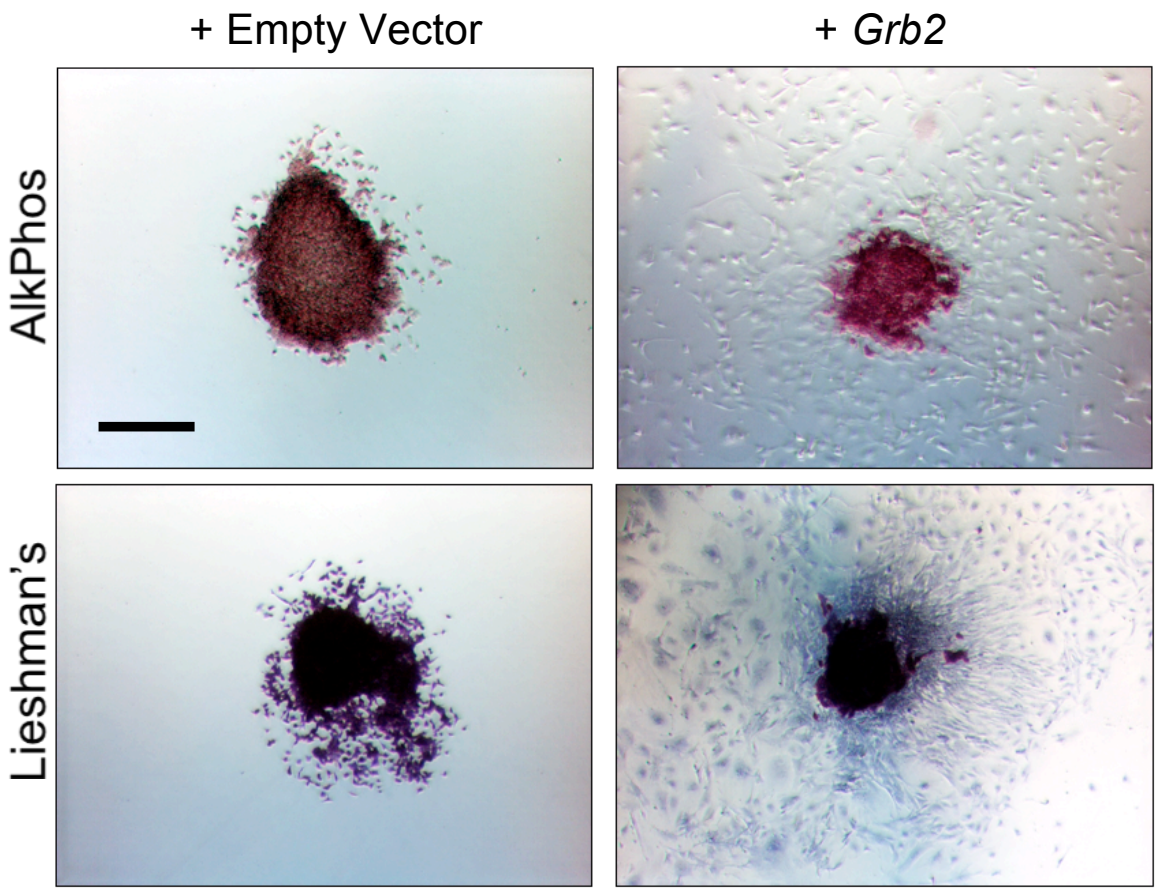


Figure 3.6 Puromycin-resistant colonies stained for alkaline phosphatase activity and Leishman's stain. *Grb2*-transfected colonies are surrounded by flat, non-AP positive cells. Scale bar = 100μm

To confirm *Grb2* was expressed in the ES cell transfectants, lysates from expanded clones were analysed by Western blotting (Fig3.7a). The level of GRB2 protein was quantified in comparison to β actin levels using Image J image analysis software (NIH) (Fig 3.7b). Restoration of GRB2 expression was confirmed in four clones. All restored clones assayed expressed *Grb2* at varying levels. *Grb2*-expressing restored clone R24 expressed GRB2 protein at a lower level than wild type. However, restored clone R28, R3 and R10 expressed GRB2 at a higher level than wild type.

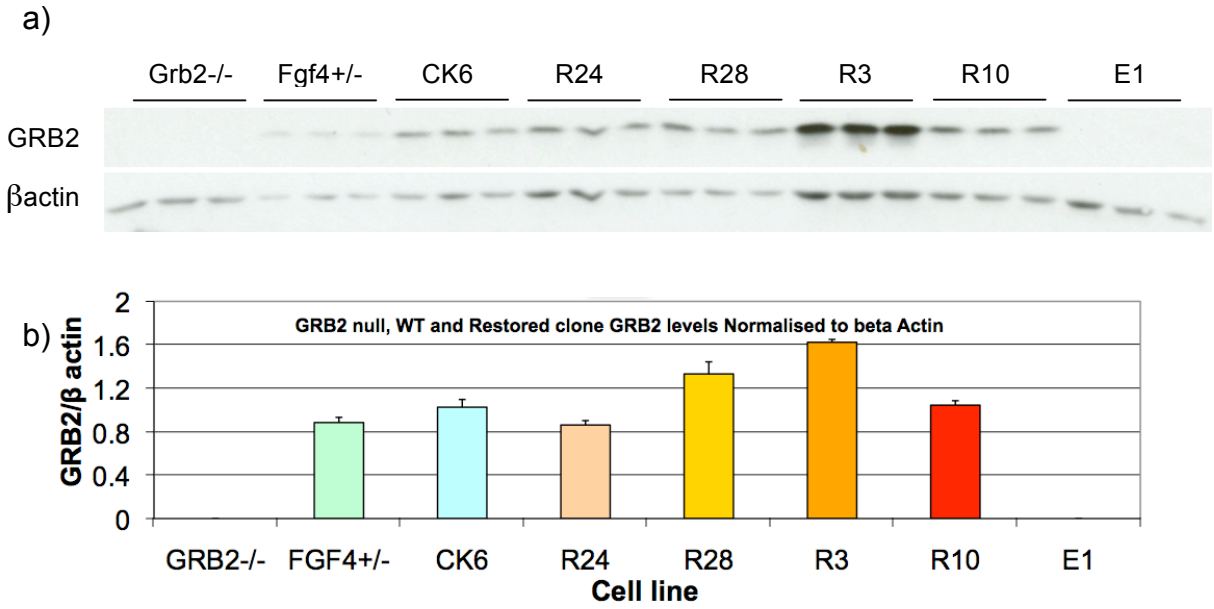


Figure 3.7 a) Levels of GRB2 protein were assessed in various R1-background ES cell lines; *Grb2*$^{-/-}$ and empty vector-transfected (E1) lines, wild type (CK6) and wild type-like (*Fgf4*$^{+/-}$) lines and four *Grb2* restored lines (R24, R28, R3 and R10). 1000 cells per sample were lysed in SDS buffer and levels of GRB2 and β actin protein were assayed by Western blot. **b)** Levels of GRB2 normalised to β actin (n=3). Analysis of Western blot by Image J image analysis software.

To assess whether restoration of *Grb2* expression was sufficient to permit growth of *Grb2*$^{-/-}$ ES cells in N2B27-LB, wild type, two *Grb2* restored lines and the parental *Grb2*$^{-/-}$ line of ES cells were cultured in N2B27-LB for three passages. At passage three, both *Grb2* restored clones demonstrated a morphology closely resembling wild type, rather than *Grb2*$^{-/-}$ cells (Fig3.8). This suggests that *Grb2* may be required for efficient growth in N2B27-LB.

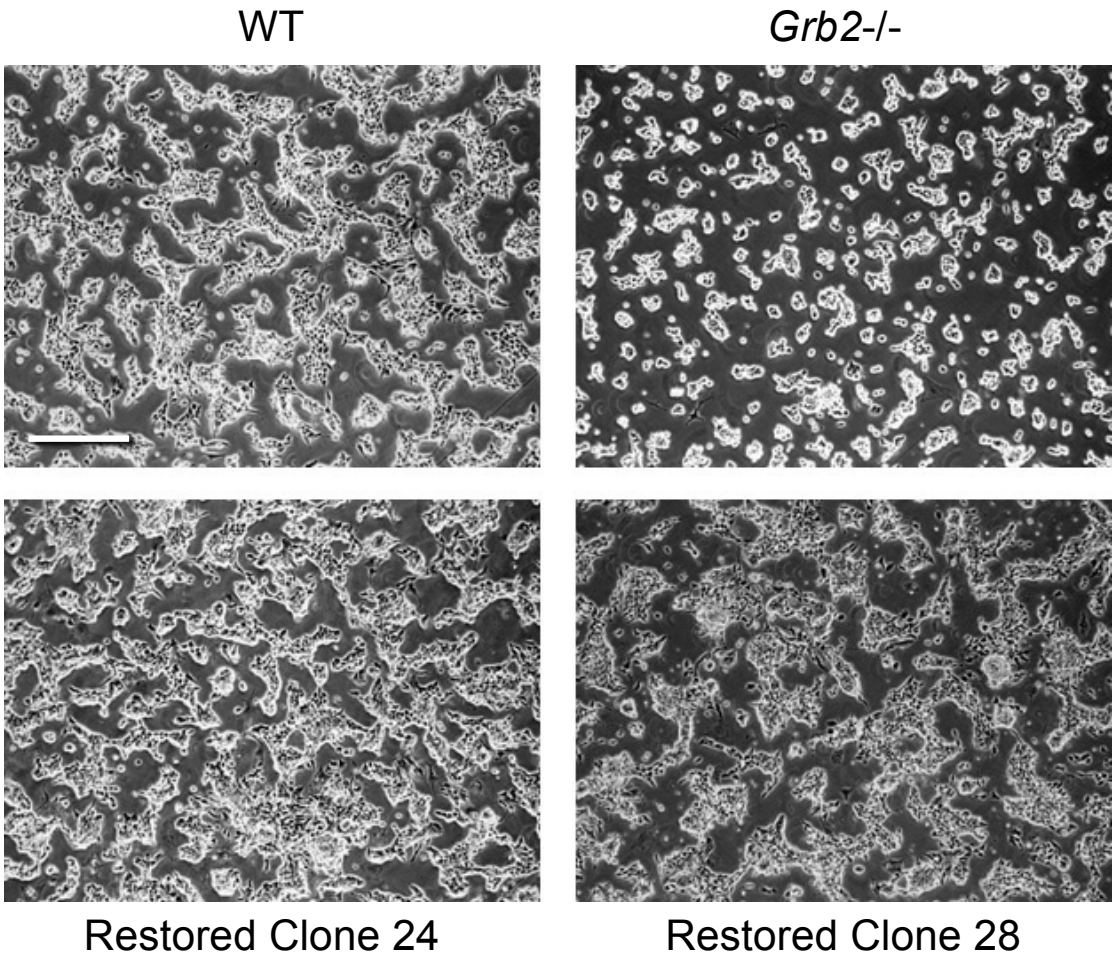


Figure 3.8 Restoration of *Grb2* expression changes the morphology of *Grb2*$^{-/-}$ ES cells in N2B27-LB. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored clones were cultured in N2B27-LB for 3 passages. Restored clone adopted a wild type-like ES cell morphology as opposed to the tight refractive colony morphology exhibited by the *Grb2*$^{-/-}$ parental line. Scale bar = 100μm.

Growth of all four cell lines in GMEM+FCS and N2B27-LB was quantified by MTT assay and by CyQuant Direct assay. The MTT assay estimates cell numbers based on the level of metabolism in a culture. This may be unsuitable for assaying *Grb2*$^{-/-}$ ES cell growth as *Grb2* may be involved in the regulation of metabolism. The CyQuant Direct assay utilises a fluorescent, live cell-permeable nucleic acid dye and a suppression dye, which is impermeable to live cells. The latter dye suppresses fluorescence in all but live, intact cells. Consequently, the CyQuant Direct assay estimates cell numbers through the measurement of DNA content of only viable cells. Technical advantages of the CyQuant Direct assay over the MTT assay include the ability to assay in the presence of phenol red, increased sensitivity enabling lower density plating and the omission of media change and lysis steps which may affect reproducibility.

$8x10^2$ (MTT) or $4x10^2$ (CyQuant Direct) wild type, *Grb2*$^{-/-}$ or *Grb2* restored ES cells were plated per well of a 96 well plate in GMEM+FCS or N2B27-LB. Replicate plates were made for each assay time point. Cells were assayed at 4, 24, 48 and 72 hours. Data was normalised to the 4-hour time point to account for potential differences in the number of cells plated at the start of the assay. The MTT assay showed no significant difference in cell numbers was measured between wild type, *Grb2*$^{-/-}$ or *Grb2* restored ES cell lines cultured in GMEM+FCS at 72 hours (Figure 3.9). However, after 72 hours culture in N2B27-LB medium, *Grb2*$^{-/-}$ cells exhibited reduced cell numbers in comparison to wild type and *Grb2* restored ES cell lines. These data suggest that the poor growth exhibited by *Grb2*$^{-/-}$ ES cells in N2B27-LB is caused by the lack of *Grb2* expression, rather than clonal variation.

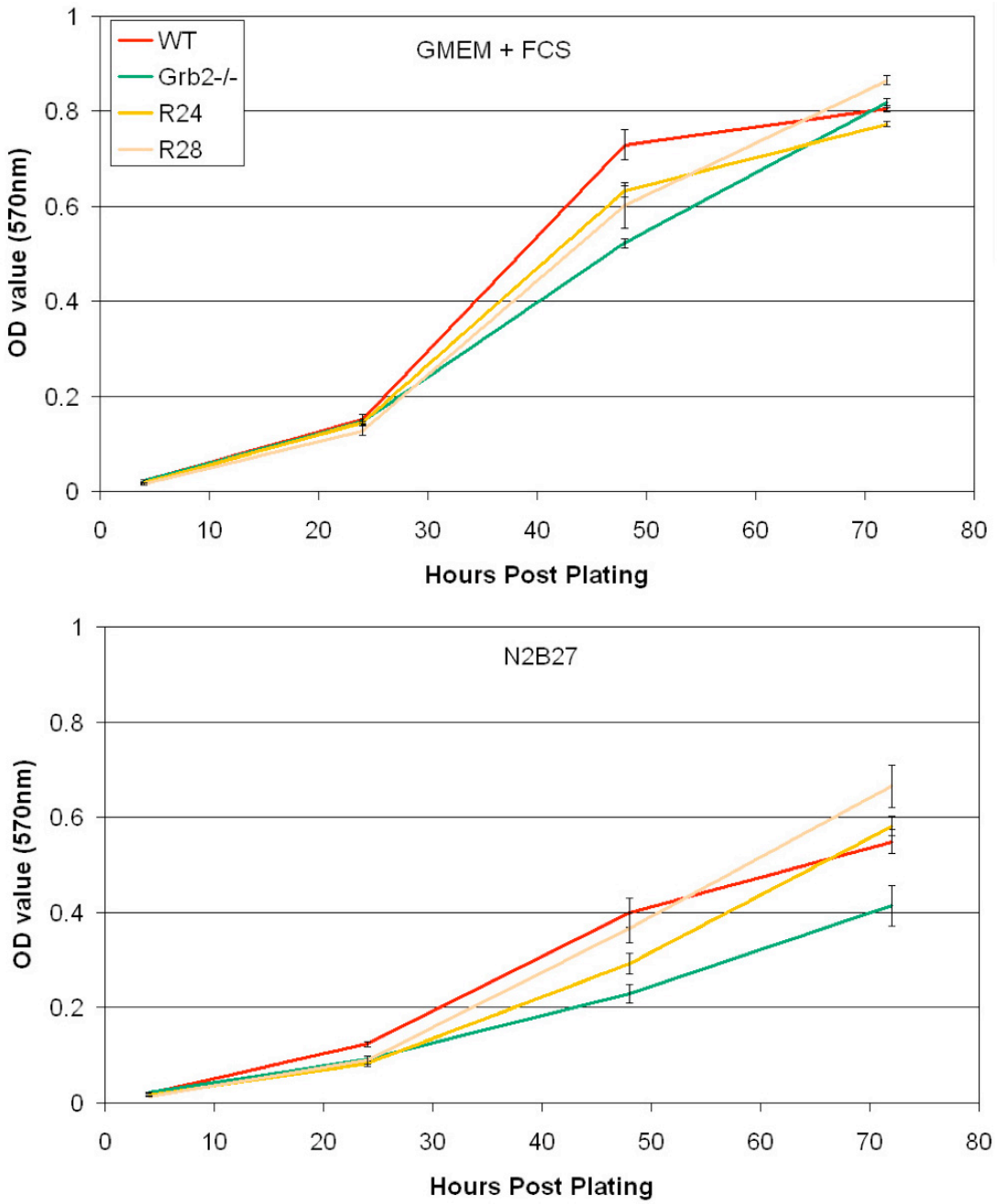


Figure 3.9 Restoration of *Grb2* expression in *Grb2*$^{-/-}$ ES increases the rate of growth in N2B27-LB medium. 8x10^2 wild type, *Grb2*$^{-/-}$ or *Grb2* restored ES cells were plated per well of a 96 well plate. Replicate plates were created for each time point. At 4, 24, 48 and 72 hours, cells were incubated with MTT substrate for 1 hour before overnight solublisation. The conversion of yellow MTT to purple formazan was quantified using a spectrophotometric plate reader with a 570nm filter. Data was normalised to the 4-hour time point (n=12 from 2 experiments).

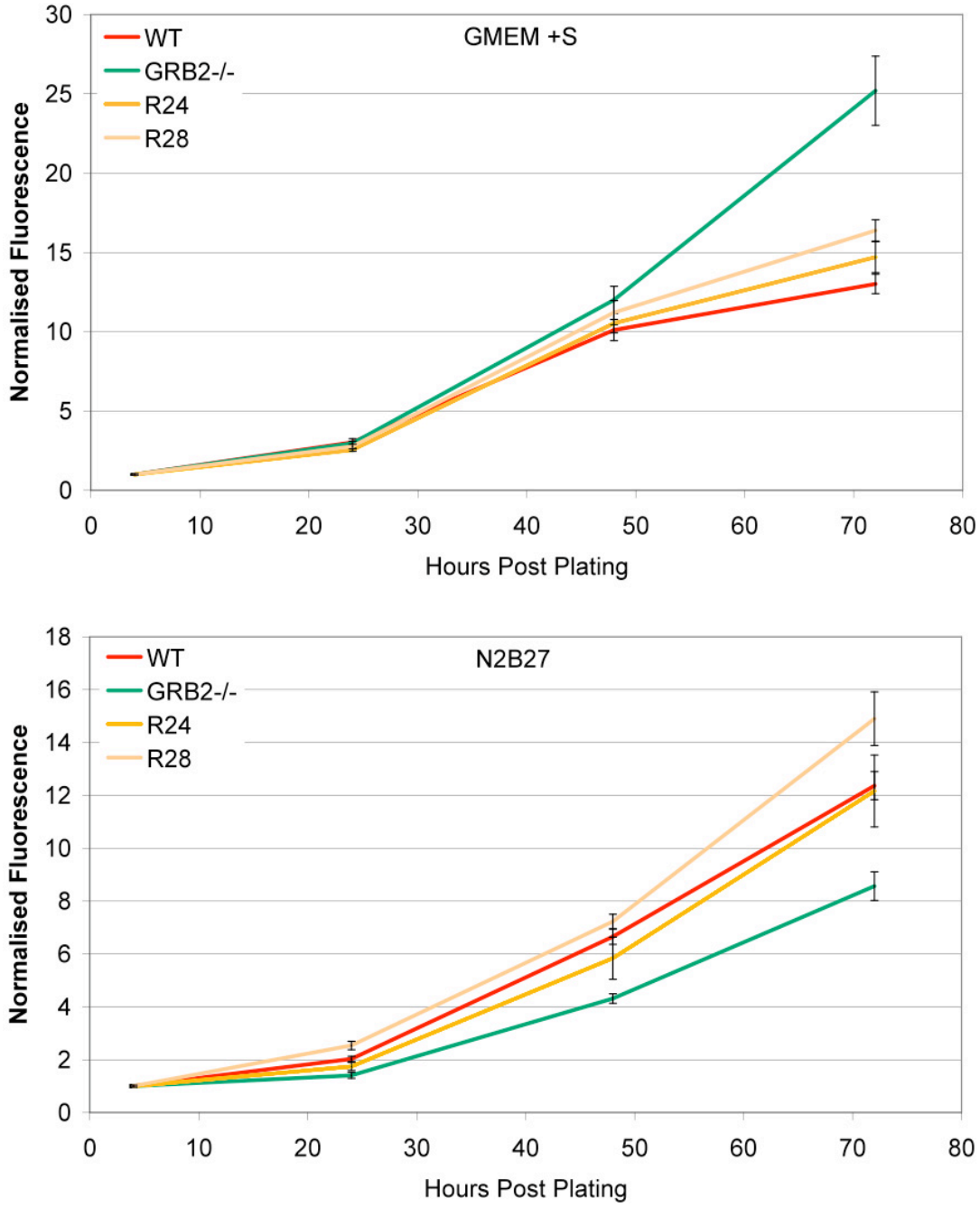


Figure 3.10 Restoration of *Grb2* expression in *Grb2*$^{-/-}$ ES increases the rate of growth in N2B27-LB medium. 4x10^2 wild type, *Grb2*$^{-/-}$ or *Grb2* restored ES cells were plated per well of a 96 well plate. Replicate plates were created for each time point. At 4, 24, 48 and 72 hours, cells were incubated with CyQuant Direct dye and suppressor for 1 hour before quantification using a fluorometric plate reader with a 488/520nm filter. Data was normalised to the 4-hour time point (n=18 from 3 experiments).

Both proliferation assays showed a significant (MTT: $p<0.01$; CyQuant Direct: $p<0.001$ by student's T-test) growth difference between wild type and *Grb2*$^{-/-}$ ES cells in N2B27-LB medium as demonstrated previously. *Grb2*$^{-/-}$ cells appeared to grow very rapidly in GMEM+FCS but this is contradicted by the result of the cell counting and MTT assay, making this result difficult to interpret. In comparison to *Grb2*$^{-/-}$ cells, both *Grb2* restored ES cell lines showed significantly (MTT: R24 $p<0.01$; R28 $p<0.001$; CyQuant Direct: R24 $p<0.05$; R28 $p<0.001$ by student's T-test) increased cell numbers compared when cultured in N2B27-LB medium. Taken together, the restoration of typical ES colony morphology and growth in *Grb2* expressing clones confirms that *Grb2* is required for growth in N2B27-LB medium.

3.2.3 Investigating the Cause of Poor Growth in *Grb2*$^{-/-}$ ES Cells.

The previous experiments show that *Grb2*$^{-/-}$ ES cells grow poorly in N2B27-LB medium. Although a slower growth rate was demonstrated, the cause of this defect was not clear. This could be due to a number of reasons, including poor cell survival and slower proliferation. To elucidate the growth deficiency we examined the extent of cell death in the cultures, cell adhesion properties and the cell cycle distribution of wild type, *Grb2*$^{-/-}$ and *Grb2* restored lines. Finding the cause of poor growth may reveal the process *Grb2* is involved in and will aid in elucidating the molecular mechanism by which *Grb2* mediates efficient growth of ES cells.

3.2.3.1 *Grb2*$^{-/-}$ ES cells Do Not Undergo Increased Apoptosis in N2B27-LB.

Apoptosis is a carefully controlled cellular program mediated by a series of molecular events resulting in the death of a cell (Kerr *et al*., 1972). Loss of membrane asymmetry is a very early event in apoptosis. In viable cells, phosphatidyl serine lipid residues are mainly found on the inner, cytosolic membrane leaflet (Verkleij *et al*., 1973). Some spontaneous switching to the outer leaflet occurs, but this is corrected by the enzyme, Flippase (Bretscher, 1973). At the onset of apoptosis, phosphatidyl serine residues are not translocated back to the inner leaflet (Fadok *et al*., 1992) and this change

can be detected using Annexin V (Vermes *et al*., 1995). Annexin V is a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidyl serines. Conjugated with a fluorochrome such as FITC, it can be used to label cells with exposed phosphatidyl serines residues. Loss of membrane integrity is a later event in apoptosis and necrosis (Kerr *et al*., 1972) and can be measured using a live cell-impermeable nucleic acid stain such as propidium iodide stain.

To investigate the levels of apoptosis in *Grb2*$^{-/-}$ ES cells, the proportion of apoptotic (annexin V positive) cells was measured. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were cultured in GMEM+FCS or N2B27-LB up to passage 3. Cells were harvested and incubated with FITC-conjugated annexin V and propidium iodide before analysis by flow cytometry. Four cell populations were defined and gated using unstained, annexin V, PI and dual stained wild type control samples. Despite decreased proliferation, *Grb2*$^{-/-}$ ES cells did not exhibit an increase in apoptosis in N2B27-LB medium (Fig 3.11a/b). There was no significant difference ($p=0.25$) in apoptotic cells compared to wild type and significantly fewer apoptotic cells in comparison to both restored lines (R24 $p<0.05$; R28 $p<0.05$).

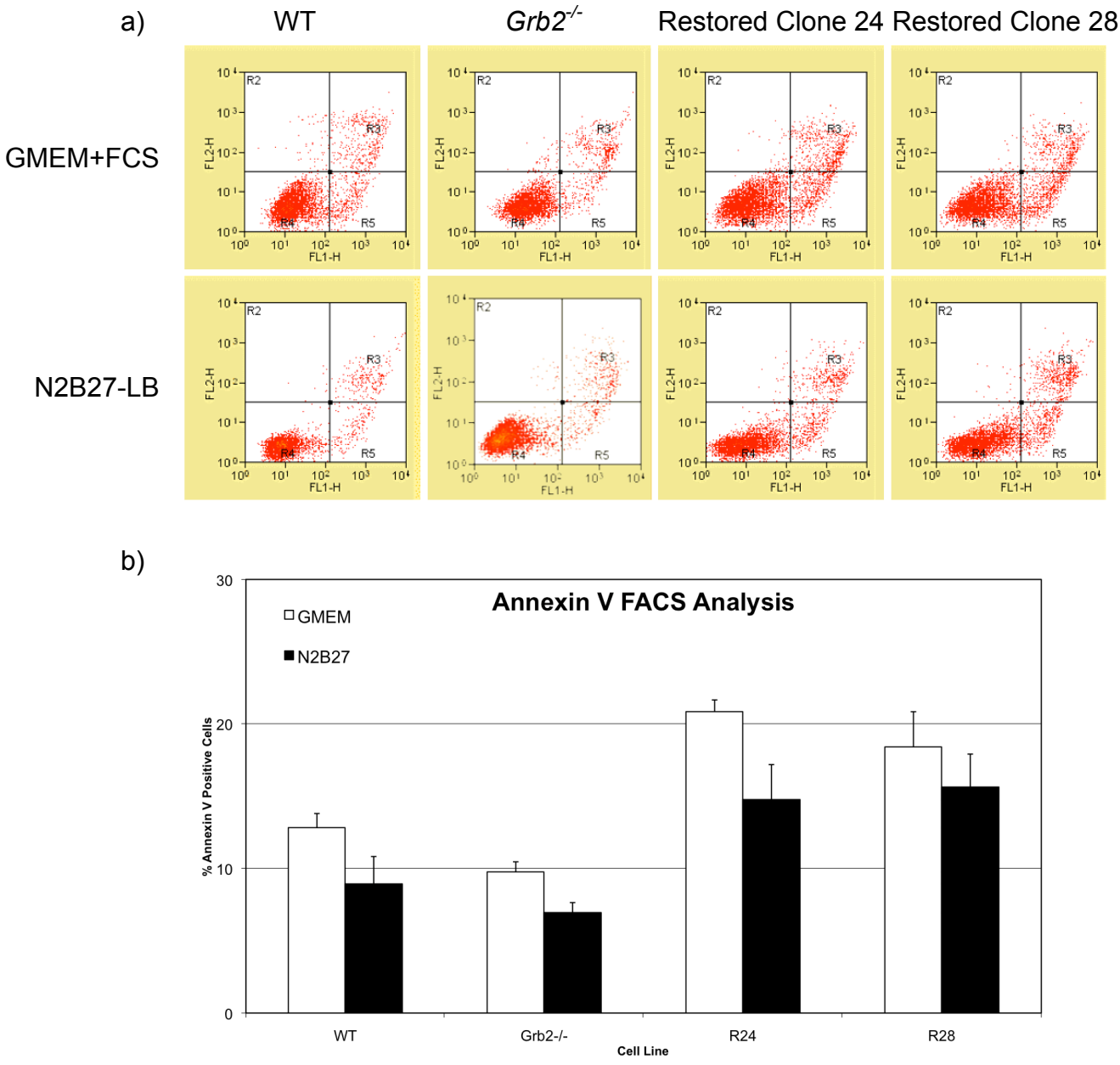


Figure 3.11 Poor growth of *Grb2*$^{-/-}$ ES cells is not accompanied by increased apoptosis in N2B27-LB. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were cultured in GMEM+FCS or N2B27-LB for 3 passages. Cells were incubated with FITC-conjugated annexin V and propidium iodide before analysis by flow cytometry. a) Typical scatter plots. Y-axis: propidium iodide. X-axis: annexin V-FITC. b) Comparison of apoptotic (annexin V positive) populations (n=4 from 2 experiments).

3.2.3.2 *Grb2*$^{-/-}$ ES cells Have No Defect in Initial Cell Adhesion.

Grb2$^{-/-}$ ES cells grow in tight, refractive colonies in N2B27-LB and some of these colonies detach from the tissue culture surface. *Grb2* is associated with integrin signalling (Schlaepfer *et al.*, 1998), therefore it was hypothesised that the decrease in cell numbers was due to a defect in adhesion, which is overcome in the presence of serum. To address this issue, an adhesion assay was performed. Wild type, *Grb2*$^{-/-}$ and *Grb2* restored ES cell lines were cultured for two passages in N2B27-LB. Cells were harvested and re-plated in replicate cultures in N2B27-LB. At 15, 30 and 60 minutes, wells were gently washed. The remaining, adherent cells were enzymatically dissociated from the culture surface and counted. At the 1 hour time point, a significantly greater number of *Grb2*$^{-/-}$ ES cells were attached to the culture surface than both wild type ($p<0.05$) and *Grb2* restored cells ($p<0.05$) (Fig 3.12). There was no significant difference between wild type and *Grb2* restored cells. This suggests *Grb2*$^{-/-}$ ES cells exhibit altered but not defective adhesion properties, at least in the initial stages of plating.

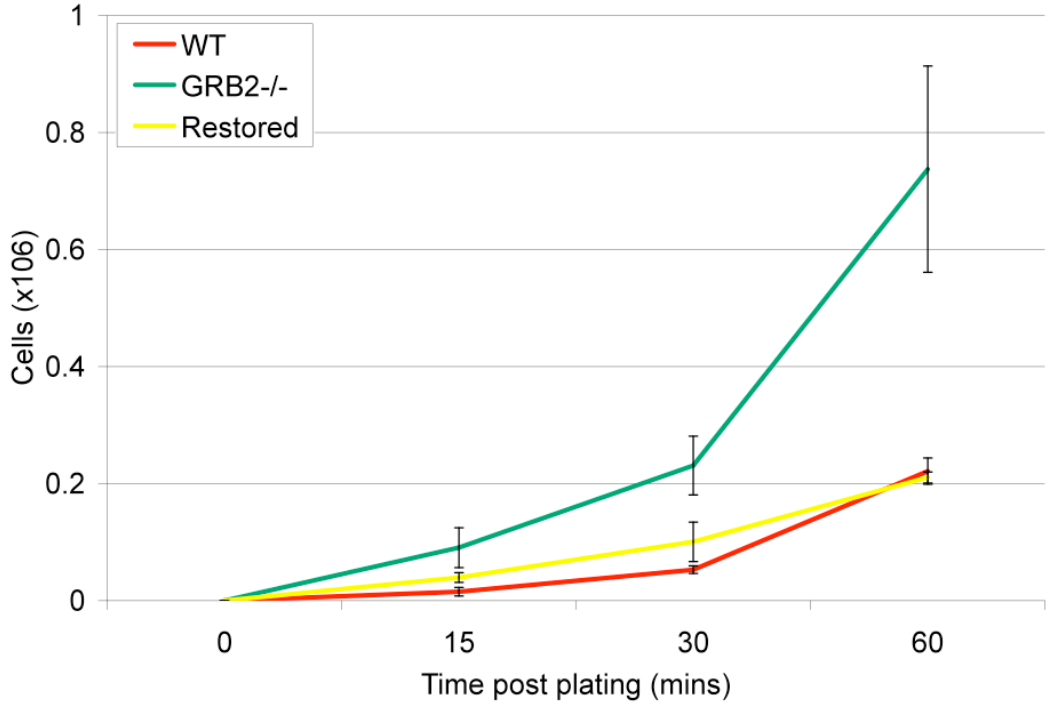


Figure 3.12 *Grb2*$^{-/-}$ ES cells show no defect in initial attachment to substrate. Wild type, *Grb2*$^{-/-}$ and a *Grb2* restored ES cell lines were cultured for two passages in N2B27-LB. Cells were harvested and re-plated in replicate cultures in N2B27-LB. At 15, 30 and 60 minutes, wells were gently washed. The remaining, adherent cells were enzymatically dissociated and counted (n=3 from 3 experiments).

3.2.3.3 Adhesion and Spreading of *Grb2*$^{-/-}$ ES Cell Colonies Is Improved with Alternative Substrates

Although *Grb2*$^{-/-}$ cells adhere well in the initial stages of plating, the morphology of the colonies in N2B27-LB suggests aberrant adhesion occurs upon colony formation. In order to test the adhesive properties of *Grb2*$^{-/-}$ ES cell colonies, cells were plated onto a range of substrates.

Based on morphology, little improvement in colony spreading was seen when cells were plated on collagen-coated, serum-coated and uncoated tissue culture plastics (Fig 3.13). However, *Grb2*$^{-/-}$ cells demonstrated a more typical ES cell-like morphology when plated on fibronectin and laminin. Colonies were no longer as tight and refractive and cellular boundaries were visible.

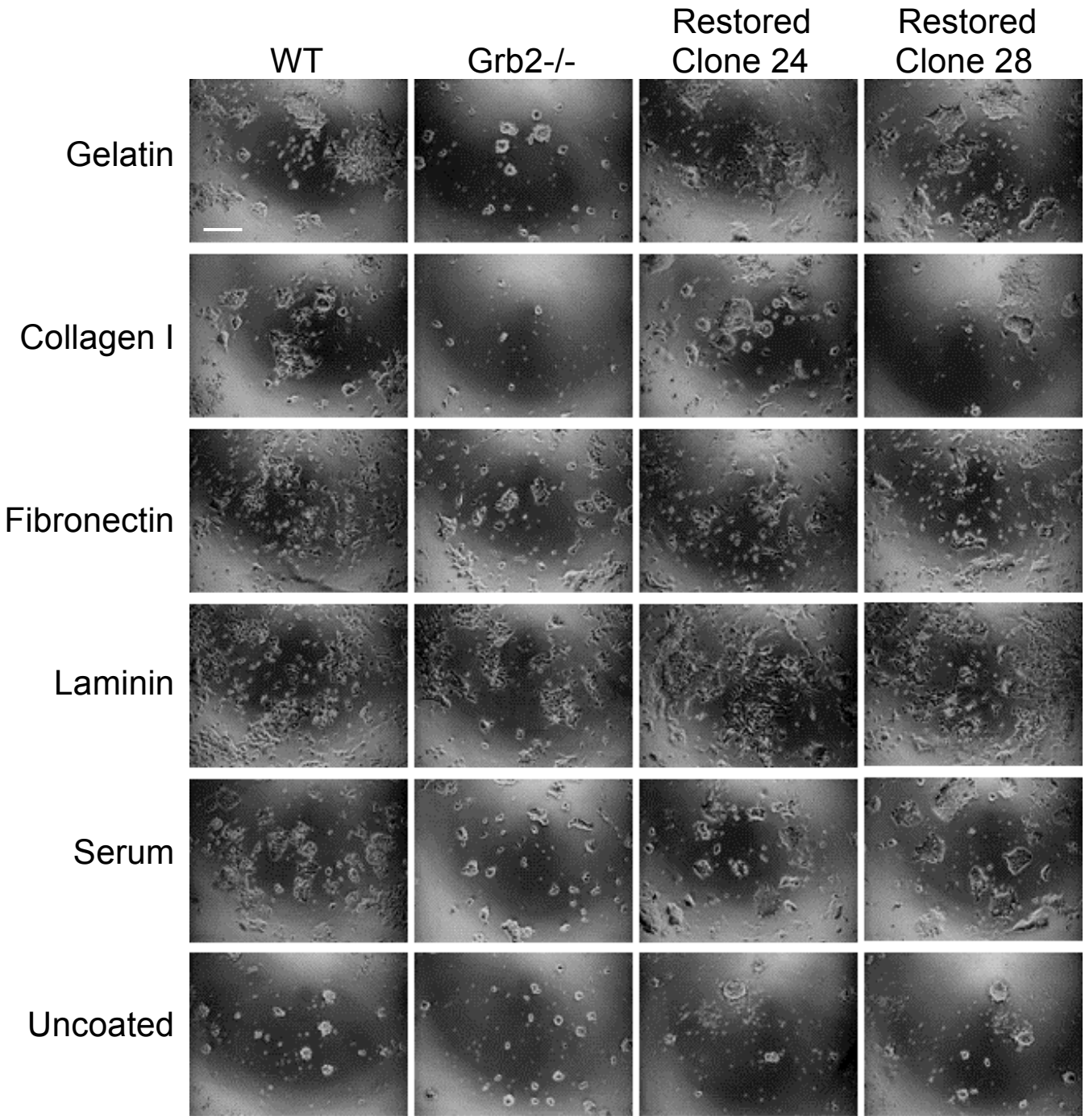


Figure 3.13 *Grb2*$^{-/-}$ colony spreading is improved upon plating on alternative substrates. Wild type, *Grb2*$^{-/-}$ and a *Grb2* restored ES cell lines were cultured on various substrates in N2B27-LB. Scale bar = 100μm.

To quantify the effects of alternative substrates on ES cell growth, a CyQuant Direct proliferation assay was performed (Fig 3.14).

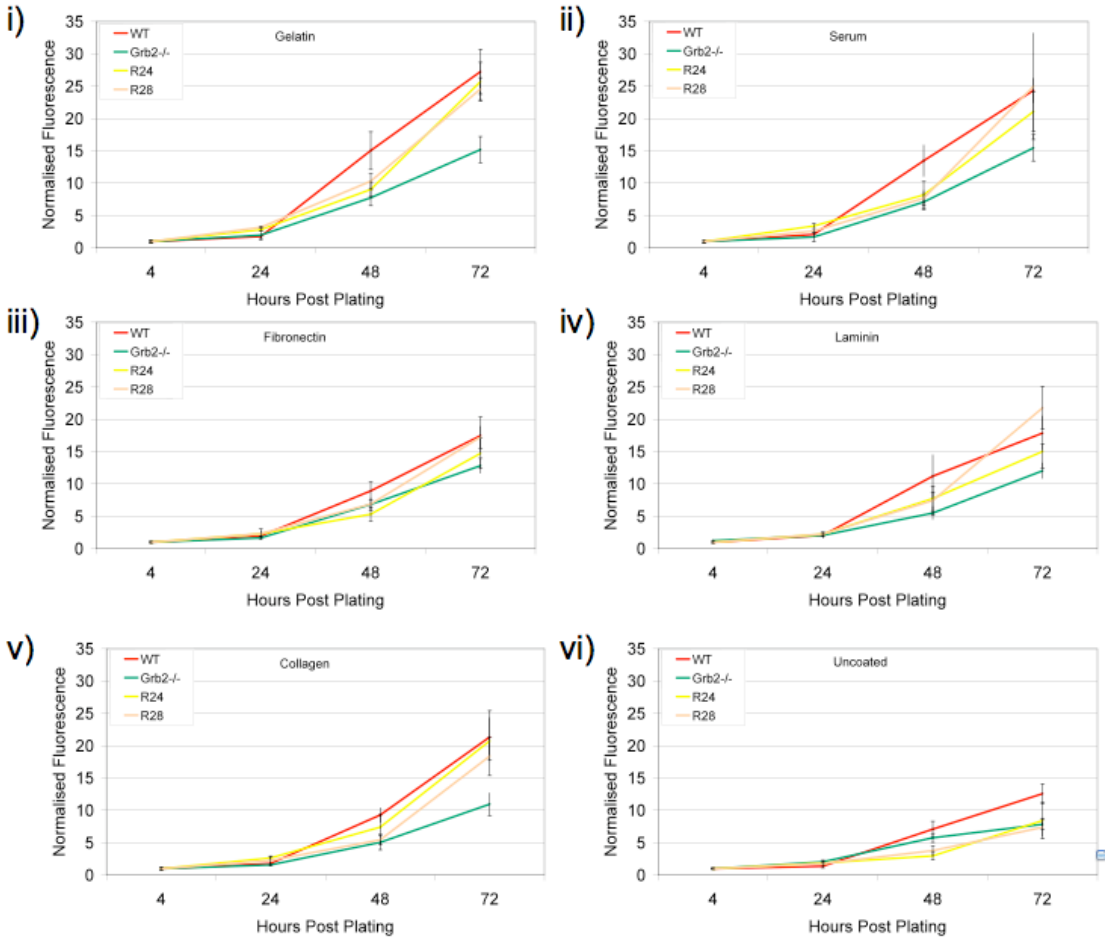


Figure 3.14 Plating on alternative substrates does not improve growth rate. $4x10^2$ wild type, *Grb2*$^{-/-}$ or *Grb2* restored ES cells were plated per well of a 96 well plate on various substrates. Replicate plates were created for each time point. At 4, 24, 48 and 72 hours, cells were incubated with CyQuant Direct dye and suppressor for 1 hour before quantification using a fluorometric plate reader with a 488/520nm filter. Data was normalised to the 4-hour time point (n=12 from 2 experiments)

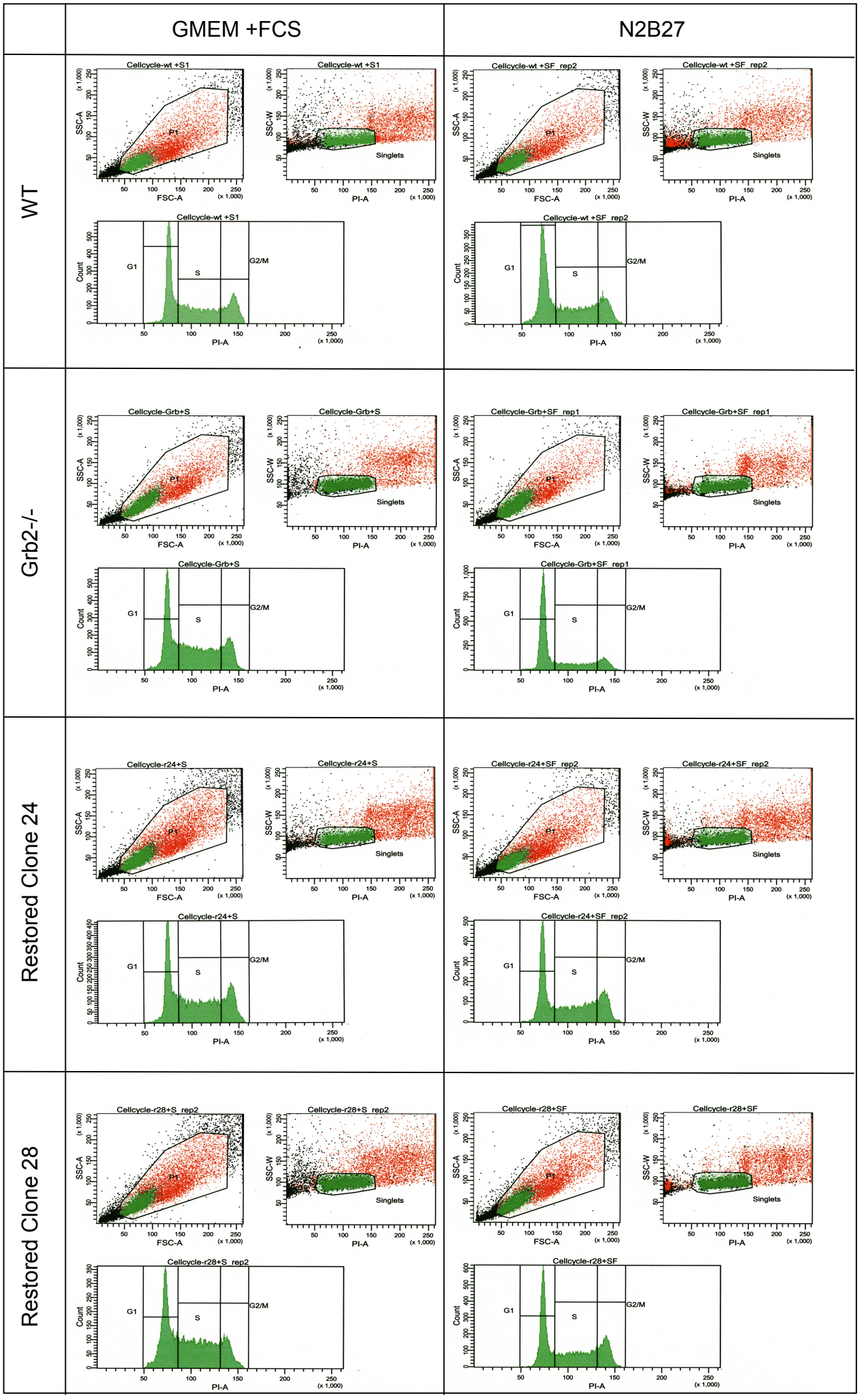
In comparison to gelatin, only *Grb2*$^{-/-}$ cells cultured on uncoated plastic resulted in a significant ($p<0.01$ by student's T-test) reduction in proliferation at the 72 hour time point. All other coatings did not significantly impact on the proliferation rate of *Grb2*$^{-/-}$ cells.

3.2.3.4 *Grb2*$^{-/-}$ ES Cells Exhibit an Abnormal Cell Cycle Distribution in N2B27-LB.

The evidence presented so far suggests *Grb2*$^{-/-}$ ES cells are not simply lost through apoptosis or poor adhesion during culture. Another explanation could be that the cells are simply proliferating more slowly in N2B27-LB media. The rate of cellular growth in all cell types is governed by the speed at which cells complete the cell cycle. Unlike somatic cells, ES cells have a particularly short G1 phase, with more cells in S phase cells (Savatier *et al*., 1996).

To investigate the cell cycle distribution of cells in GMEM+FCS and N2B27-LB media, the flow cytometry profiles of PI-stained cells were compared (Fig 3.15a/b). In GMEM+FCS medium, wild type, *Grb2*$^{-/-}$ and two *Grb2* restored cell lines exhibited very similar profiles. This supports data presented previously in this chapter, which showed all cell lines proliferate at a similar rate in this medium. Indeed, the cell cycle profiles exhibited a distribution typical of mouse embryonic stem cells, with the largest proportion of cells in S phase (Stead, *et al*., 2002). In N2B27-LB, the proportion of wild type and *Grb2* restored cells in G1 and S phase was similar. However, *Grb2*$^{-/-}$ cells exhibited a markedly different profile in the different media, with approximately 15% more cells in G1 phase ($p<0.01$ by student's T-test), 10% fewer cells in S phase ($p<0.005$) and 5% fewer cells in G2/M phase (p=not significant) (Fig 3.15a/b).

a)


GMEM +FCS
N2B27
WT
Grb2-/-
Restored Clone 24
Restored Clone 28
Cellcycle-wt +S1
Cellcycle-wt +SF_rep2
Cellcycle-Grb+S
Cellcycle-Grb+SF_rep1
Cellcycle-r24+S
Cellcycle-r24+SF_rep2
Cellcycle-r28+S_rep2
Cellcycle-r28+SF
SSC-A
FSC-A
SSC-W
PI-A
P1
Singlets
Count
G1
S
G2/M
(x 1,000)

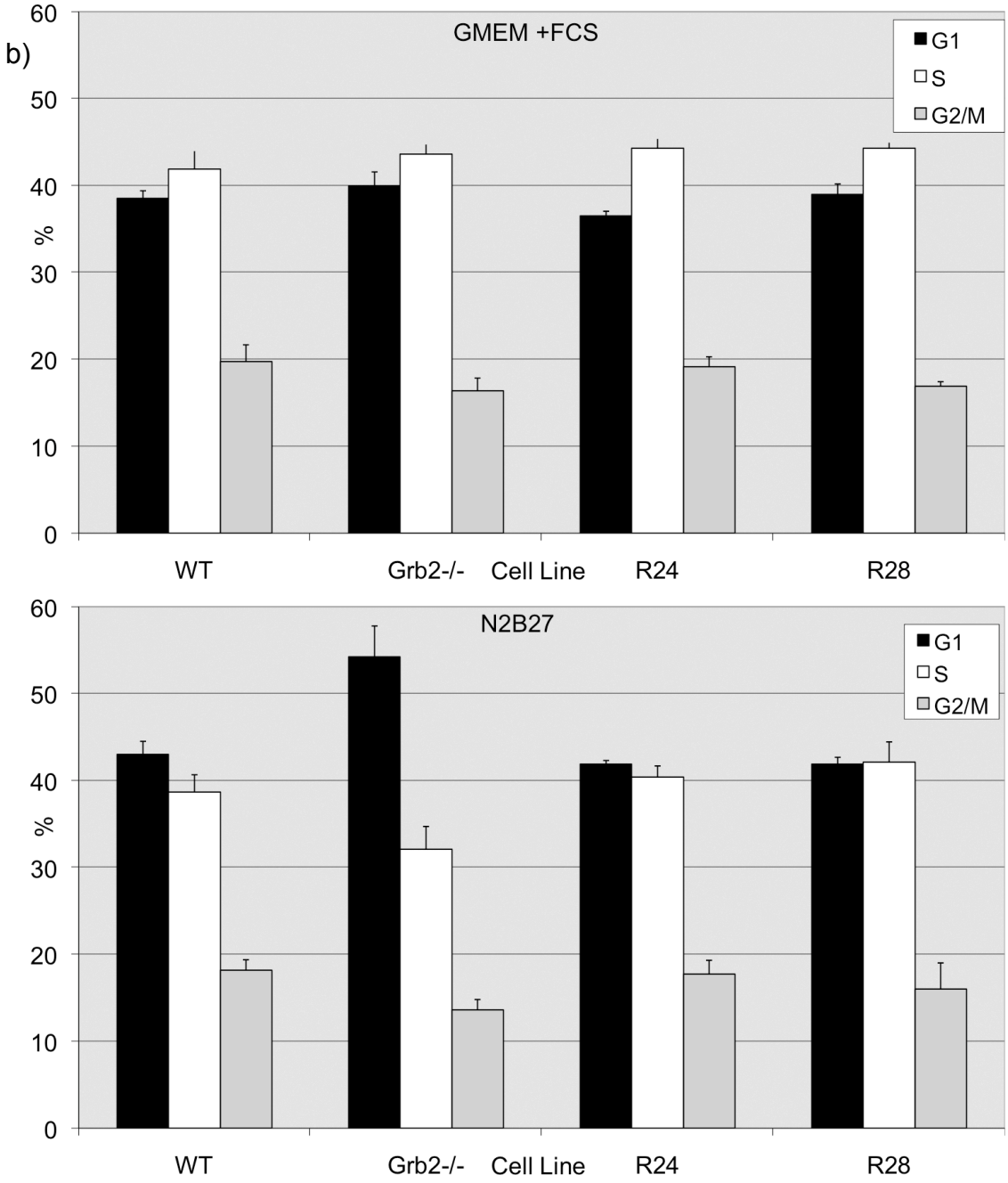


Figure 3.15 *Grb2*$^{-/-}$ ES cell exhibit an abnormal cell cycle distribution. Wild type, *Grb2*$^{-/-}$ and *Grb2* restored ES cells were cultured in N2B27-LB for 3 passages before fixation and PI staining followed by flow cytometry analysis a) Typical scatter plots. b) Mean percentage of population in G1, S or G2/M phase (n=6 from 3 experiments).

3.3 Discussion

This chapter has established for the first time that *Grb2* plays a wider role in ES cell biology than might have been anticipated, based on previous reports. These results suggest that in addition to its role in differentiation, *Grb2* is also required for efficient growth in defined culture conditions in N2B27-LB. This was demonstrated by the poor growth phenotype exhibited by *Grb2* ES cells in N2B27-LB media. Poor growth is characterised by a decrease in cell numbers confirmed by cell counting, metabolic and DNA-based proliferation assays.

Grb2$^{-/-}$ ES cells express high levels of ES cell markers *Oct4* and Nanog. This is verified by existing studies in which *Grb2*-mediated MAPK activation was shown to downregulate Nanog (Hamazaki *et al*., 2006). This may suggest low levels of MAPK activity in *Grb2*$^{-/-}$ ES cells in N2B27-LB medium.

Grb2 was confirmed as the essential component missing from the cells as restoration of *Grb2* expression resulted in improved ES cell morphology and growth rate, which closely resembled the wild type phenotype.

It was determined that *Grb2*$^{-/-}$ ES cells were not undergoing increased apoptosis and initial attachment of the cells to the tissue culture surface was not defective. When plated on laminin or fibronectin, the morphology of *Grb2*$^{-/-}$ cells in N2B27 changed to resemble wild type cells; although proliferation data suggested growth had not been rescued. This suggests that morphology and proliferation are not linked under these conditions.

Grb2$^{-/-}$ ES cell colonies attach poorly to the gelatin-coated tissue culture surface, however, the tight, refractive colonies suggest cell-cell adhesion may be altered. This implies the poor growth phenotype may be characterised by changes in adhesion properties. This may indicate integrin signalling is disrupted. Integrins are receptors required for both cell-extracellular matrix (ECM) and cell-cell interactions. They consist of different isoforms of α and β subunits enabling a number of combinations that confer ligand-binding specificity to the resulting heterodimers. Insoluble forms of

ligands including laminins, fibronectin and collagens can be used as substrates to facilitate cell adhesion through the formation of focal contacts between integrins and the ECM. Adhesion to substrates can result in profound effects on cell growth (Zhu *et al*., 1996), death (O'Brien *et al*., 1996), shape and migration (Bauer *et al*., 1984).

As well as adhesion molecules, integrins are also instigators of cell signalling. Ligand-bound integrins have been shown to induce tyrosine phosphorylation of proteins, including focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase co-localised with and activated by integrin receptors at sites of cell-ECM interaction.

The importance of the ECM and integrin signalling is highlighted by the embryonic lethality of fibronectin or $FAK^{-/-}$ mice. Fibronectin null embryos are able to implant, gastrulate and form neural folds but the anterior posterior axis does not reach full length and the neural tube is deformed (George *et al*., 1993). Mesoderm-derived tissue suffers severe defects; notochord and somites are not formed and the heart is poorly developed. Defects in the yolk sac, extraembryonic vasculature and amnion are also noted. Ilic *et al*. (1995) reported strikingly similar phenotype in $FAK^{-/-}$ embryos, suggesting fibronectin-integrin interactions are mediated by FAK at this stage of development.

Grb2 mediates MAPK signalling by various integrin-related mechanisms. Alpha1 integrin activates MAPK via Fyn, Shc and *Grb2* (Wary *et al*., 1998). *Grb2*-mediated β integrin signalling can occur in a FAK-dependent manner (Schlaepfer *et al*., 1998) or by direct association with the cytoplasmic domain of β integrins (Law *et al*., 1996).

It is unlikely that the morphology of $Grb2^{-/-}$ ES cells in N2B27-LB is caused by insufficient integrin activity in the initial stages of attachment, as $Grb2^{-/-}$ cells did not exhibit any defect in adhesion within the first hour of plating. The β_1 integrin deficient ES cell phenotype supports this (Fässler *et al*., 1995). Although β_1 null ES cells exhibit an unusually tight colony morphology, similar

to *Grb2*$^{-/-}$ cells in N2B27-LB, attachment to laminin and fibronectin is impaired. This is the opposite effect to that seen in *Grb2*$^{-/-}$ ES cells, where attachment is improved on these substrates.

The improvement in adhesion and colony spreading seen on laminin and fibronectin substrates may indicate that an alternative mechanism of adhesion can be triggered to induce colony spreading in a *Grb2*-independent manner. One explanation for this could be the involvement of an alternative combination of integrin α and β subunits.

A series of events occur in the process of cell attachment. Initially, integrins mediate weak binding to RGD protein sequences found in ECM proteins and this attachment strengthens as integrin clusters are formed in the cell membrane (Gottschalk *et al*., 2004). Next, secondary attachment proteins such as talin, vinculin and α actinin accumulate at these clusters and a network of interconnected proteins is created within the cluster, which interacts with the cytoskeleton (Cluzel *et al*., 2005).

The secondary events linking integrins to the cytoskeleton may be affected in *Grb2*$^{-/-}$ ES cells. Chen *et al*. (1994) showed cytochalasin D, a potent inhibitor of actin polymerization, eradicates MAPK activation by integrins, which is likely to be mediated by *Grb2*.

Grb2 also interacts with the cytoskeleton via direct interactions with two cytoskeletal proteins. Caldesmon is a calmodulin and actin binding protein (Boerner *et al*., 2003) and Magicin is a Merlin and *Grb2* interacting cytoskeletal protein (Wiederhold *et al*., 2004).

These data implicate *Grb2* in the molecular mechanisms of cell attachment, which may contribute to the poor growth phenotype exhibited in N2B27-LB. This could be investigated further using peptides and antibodies that specifically block integrin interactions. For example, ES cells express $\alpha_v\beta_5$ integrins (Lee *et al.,* 2009) which bind to vitronectin, a major cell attachment-promoting ECM protein found in serum (Hayman *et al.,* 1985). Culturing wild type ES cells in N2B27-LB medium or *Grb2*$^{-/-}$ cells in GMEM+FCS medium in

the presence of α_v and β_5 integrin-specific antibodies may recreate the poor growth phenotype described in this chapter, whereas no effect would be seen on *Grb2*$^{-/-}$ cells in N2B27-LB medium. Multiple combinations of integrin subunits can be investigated to identify the integrins responsible. Comparing the integrin expression profiles of *Grb2*$^{-/-}$ cells in N2B27-LB and GMEM+FCS media by FACS analysis may also reveal the underlying cause of abnormal adhesion.

Changes in the cell cycle distribution indicate that growth may be inhibited due to slow progression through the G1 checkpoint. This proposal is supported by the current understanding of the role of MAPK in cell cycle progression. MAPK activation has been linked with increased cyclinD1 expression (Terada *et al*., 1999) and the assembly of the cyclinD1/Cdk4+6 complex, which is known to upregulate genes required for G1/S transition (Ewen, 2000). However, cyclinD is not thought to play a role in the ES cell cycle due to its low level of expression (Savatier *et al*., 1996; Jirmanova *et al*., 2002). This may suggest that trace levels of cyclinD are sufficient for efficient progression through G1 under defined conditions.

Another explanation could be that *Grb2* might regulate the cell cycle via a direct interaction with p27. p27 is a cell cycle inhibitor expressed at low levels by ES cells (Stead *et al*., 2002). Upon binding, GRB2 has been shown to facilitate the degradation of p27 (Sugiyama *et al*., 2001). In the absence of *Grb2*, levels of p27 may accumulate and inhibit the progression of the cell cycle.

These data indicate *Grb2* does regulate ES cell growth, when compensatory mechanisms that are normally provided by serum, are unavailable. *Grb2* is required for normal adhesion to a gelatin substrate and plays a distinct role in efficient progression through the cell cycle (Fig3.16).

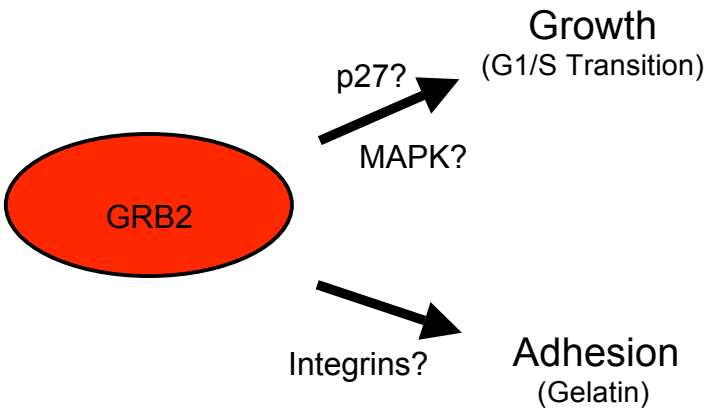


Figure 3.16 Schematic of novel findings described in this chapter. *Grb2* is required for efficient ES cell growth and attachment in N2B27-LB medium.

CHAPTER 4

Grb2 Signalling is Required for Efficient Growth in N2B27-LB Medium.

4.1 Introduction

The previous chapter described the novel finding that *Grb2* plays an important role in the regulation of mouse ES cell growth. *Grb2*$^{-/-}$ ES cells grow poorly in N2B27-LB. The data suggest poor growth is caused by a defect in proliferation as no increase in apoptosis was measured while there was an accumulation of cells in G1 phase. This chapter will describe an investigation into signalling pathways downstream of *Grb2* responsible for impaired growth of *Grb2*$^{-/-}$ ES cells in N2B27-LB media.

4.1.1 Signals Immediately Downstream of *Grb2*

The GRB2 adapter protein is ubiquitously expressed and interacts with a number of downstream effectors, some of which are tissue specific. The N-terminal SH3 domain of GRB2 predominantly binds to the proline rich domain of Son-of-Sevenless (SOS) (Wittekind *et al*., 1997) whilst the C-terminal SH3 domain binds to a similar domain in GAB1 (Ong *et al*., 2001). Both SOS and GAB1 can bind to GRB2 simultaneously, enabling concurrent activation of pathways downstream of SOS1 and GAB1.

GRB2 also directly associates with VAV (Ye and Baltimore *et al*., 1994), a proto-oncogenic protein expressed by the haematopoietic lineage. VAV is a guanine exchange factor involved with the activation of RhoGTPase, a key regulator of cell proliferation, shape and survival (reviewed in Etienne-Manneville and Hall, 2002). GRB2 binding facilitates translocation of VAV to the membrane in response to stimulation of the T cell antigen receptor and subsequent tyrosine phosphorylation of Linker for Activation of T cells (LAT).

Wiskott-Aldrich syndrome protein (WASp) is a scaffold protein that binds strongly with GRB2 N-terminal SH3 domain and more weakly to the C-

terminal SH3 domain (She *et al*., 1997). GRB2 binds to phosphotyrosine residues presented by the activated EGF-receptor, translocating WASp from the cytoplasm to the membrane where it plays a role in the regulation of the actin cytoskeleton of T cells through its interaction with the actin-related protein 2/3 (Arp2/3) complex. No phenotype was reported in $WASp^{-/-}$ ES cells (Cotta-de-Almeida, *et al*. 2007), however, $WASp^{-/-}$ mice were defective in T cell development.

4.1.2 MAPK

The mitogen-activated protein kinase (MAPK) cascade encompasses a series of sequentially activated serine/threonine kinases. In response to phosphotyrosine residues presented by active RTKs and docking proteins, GRB2 facilitates the translocation of SOS to the membrane. SOS mediates the exchange of GDP for GTP on Ras, a small GTP binding protein. Active, GTP-bound Ras initiates the MAPK cascade of Raf, MEK and ERK activation (Fig 4.1a). Signalling downstream of ERK includes both cytoplasmic targets (Sturgill *et al*., 1988) and nuclear transcription factors (Chen *et al*., 1992).

There are many signals downstream of ERK. More than 150 ERK substrates have been identified to date and this number is expected to increase (Krishna and Narang, 2008). The best studied downstream target of ERK is E-twenty six-like gene 1 (*Elk1*). *Elk1* is a component of the ternary complex factor (TCF) subfamily, forming a ternary complex upon binding the serum response factor (SRF) and the serum response element (SRE). Phosphorylation by ERK increases the DNA binding capacity of *Elk1* which eventually results in the upregulation of proto-oncogene *c-Fos*.

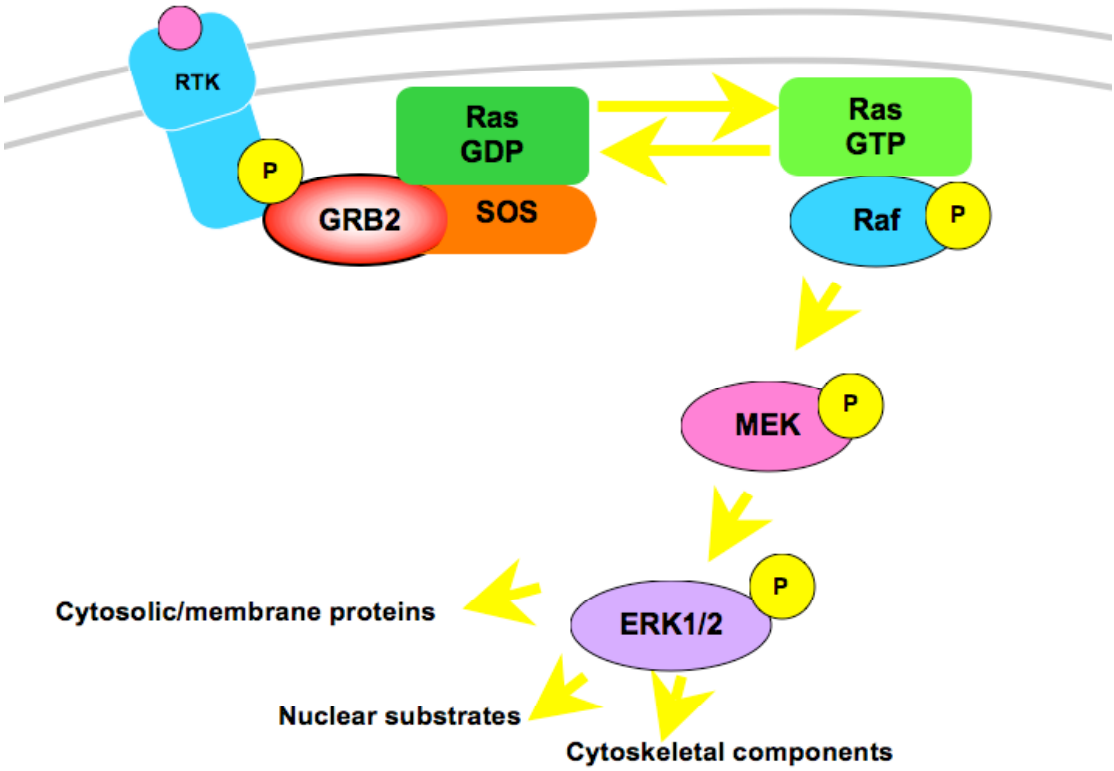


Figure 4.1 The MAP-kinase pathway. Receptor tyrosine kinases or scaffold proteins present phosphotyrosine residues upon activation. The SH2 domain of adaptor protein GRB2 enables binding to these phosphotyrosines. GRB2 also binds to Son of sevenless (SOS) through a SH3 domain. As the GRB2/SOS complex is brought to the membrane via a phosphotyrosine interaction, SOS is activated and can carry out its function as a guanine exchange factor of Ras, a small GTP binding protein. SOS exchanges GDP for GTP and Ras is able to activate Raf, a kinase of MEK (MAPK/ERK kinase) which in turn is a kinase of ERK (extracellular signal-regulated kinase). Once activated, an ERK dimer regulates cytosolic targets, cytoskeletal proteins and can also enter the nucleus to regulate various transcription factors.

Burdon *et al*. (1999) demonstrated that suppression of MAPK signalling, specifically MEK signalling, inhibited differentiation in ES cells. This work was later developed into the model wherein ERK activation shifts pluripotent ES cells into a reversible state where cells are receptive to differentiation signals (Kunath *et al*., 2007). ES cells under MEK inhibition are described as 'Ground State' ES cells, indicating their status as a homogeneous, pluripotent population of ES cells (Ying *et al*., 2008).

4.1.3 PI3K

The PI3K pathway can be activated through direct receptor interactions or by an indirect mechanism. For example, PI3K/AKT is activated by PDGF via the direct recruitment of PI3K to the activated PDGF receptor (Kazlauskas *et al*., 1992). However, this is not the case for many RTKs. FGF signalling employs an intermediate docking molecule to achieve PI3K activation via GRB2 and *Grb2*-associated binding protein 1 (Gab1). The FGF-Receptor Substrate (FRS) contains numerous tyrosine residues, which are phosphorylated in response to the autophosphorylation of ligand-bound FGF-receptor (Fig 4.1B). GRB2 is recruited via its SH2 domain and serves as an adapter molecule between the receptor substrate and Gab1, which is bound to GRB2 at its C-terminal SH3 domain. Activation of Gab1 in this manner generates phosphotyrosines which recruit PI3K regulatory subunit p85 to the membrane resulting in PI3K activity.

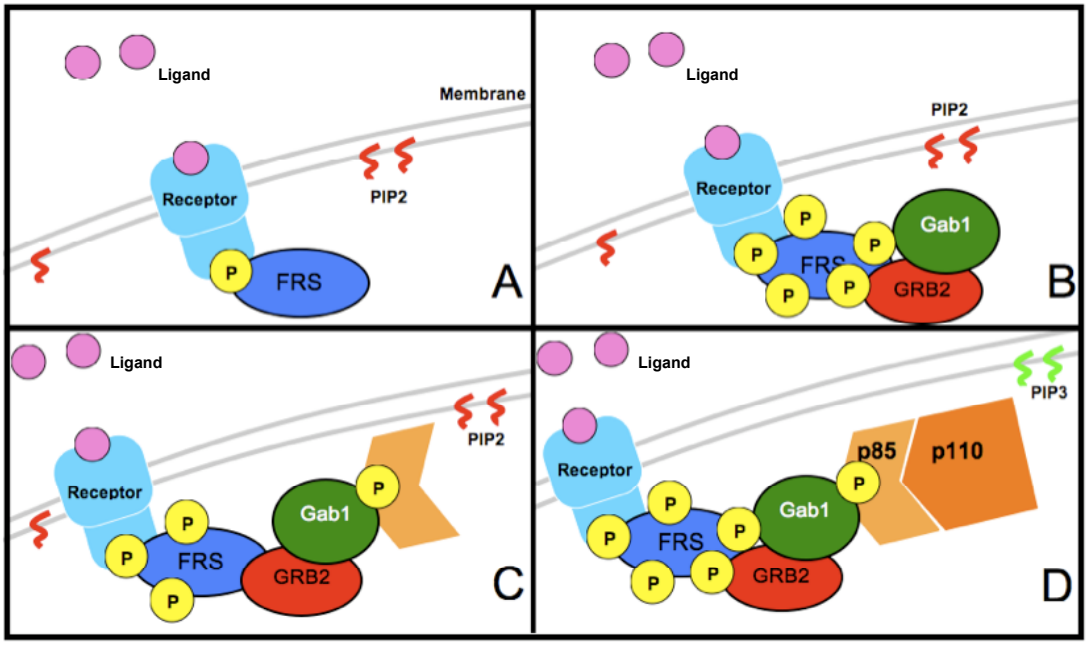


Figure 4.2 FRS-facilitated activation of PI3 kinase. A) Ligand is presented to receptor and the receptor's intracellular domain autophosphorylates, presenting a phosphotyrosine residue. FRS is then recruited to the receptor. B) Activated FRS presents many phosphotyrosine residues and the GRB2-Gab1 complex is recruited via the GRB2 SH2 domain. Gab1 recruits the regulatory subunit (here p85) of PI3K. C) The catalytic subunit (e.g. p110) is recruited to the membrane by the regulatory subunit. D) The catalytic subunit is now in close proximity to it's substrate, PIP2, which is phosphorylated to form PIP3.

PI3K has been shown to apply to many functions within the cell including proliferation, differentiation and survival. In ES cells, PI3K has been implied to play a role in both self-renewal (Paling *et al*., 2004; Watanabe *et al*., 2006) and proliferation (Jirmanova *et al*., 2002).

Many studies indicate PI3K/AKT signalling regulates the control of ES cell growth. ES cells under inhibition of PI3K activity through the activity of inhibitor LY294002 exhibit decreased proliferation, with cells accumulating in the G1 phase of the cell cycle. Also, deletion of Eras, an ES cell-specific form of Ras responsible for constitutive activation of PI3K results in poor proliferation and reduced tumourigenicity (Takahashi *et al*., 2003). Conversely, Phosphatase and Tensin Homolog (PTEN) is a negative regulator of PI3K through its function as a PIP3 phosphatase (Maehama and Dixon, 1998). Knockdown of this protein results in high levels of PIP3, essentially a hyperactivation of the PI3K signal. $PTEN^{-/-}$ ES cells demonstrate increased proliferation and enhanced tumourigenicity (Sun *et al*., 1999). Normal growth is restored upon deletion of AKT from $PTEN^{-/-}$ ES cells (Stiles *et al*., 2002), suggesting AKT is the key downstream effector of the PI3K signal in ES cells. However, no proliferative defect was reported in $PDK1^{-/-}$ ES cells (Williams, 2000). As PDK1 is responsible for the phosphorylation of AKT, it was suggested ES cells do not require AKT. This work was carried out in serum-based media, therefore it is possible that $PDK1^{-/-}$ cells survive due to an undefined factor present in the media, perhaps in a similar manner to $Grb2^{-/-}$ ES cells.

GSK3β is a major downstream effector of PI3K signalling in ES cells. This is supported by Storm *et al*. (2007) who showed inhibition of GSK3β reverses the differentiative effect of PI3K inhibition via inhibitor LY294002. However, McManus *et al*. (2005) generated ES cells expressing a form of GSK3β containing a serine-alanine substitution at residue 9. This mutation disabled GSK3β inhibition via AKT phosphorlyation without affecting self-renewal, suggesting GSK3β phosphorylation via AKT may not be required by ES cells.

GSK3β inhibition via Wnt3a remained intact in these cells, which may indicate Wnt signalling is solely responsible for GSK3β inhibition in ES cells or perhaps both pathways encompass a convergent function to keep this integral pathway under tight control.

This chapter describes experiments with the aim of defining and characterising the pathways regulated by *Grb2* that are required for efficient growth.

4.2. Results

4.2.1. AKT Signalling is Not Disrupted in *Grb2*$^{-/-}$ ES Cells

PI3K/AKT is a major signalling pathway associated with cell growth and survival (Yao and Cooper, 1995). Activation of AKT has been shown to inhibit apoptosis (Kulik *et al*., 1997) and increase protein synthesis (Gingras *et al*., 1998). Forced expression of activated AKT has been shown to support ES cell self-renewal in the absence of LIF (Watanabe *et al*., 2006). Disruption of this pathway can cause poor growth in many cell types (Fruman *et al*., 1999; Gao *et al*., 2000; Coutant *et al*., 2002).

Evidence suggests the PI3K/AKT pathway is important for cell proliferation and survival and *Grb2* can regulate PI3K/AKT signalling through its role in recruitment of Gab1 to the membrane. To investigate the status of the ES cell PI3K/AKT pathway in the absence of *Grb2*, ES cells were stimulated with FGF or IGF growth factor and the activity of the PI3K/AKT pathway was assayed biochemically. A wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated overnight, washed and starved of serum for 3 hours to reduce background signalling. After this time, cells were then exposed to either 25ng/ml FGF2 and 1μg/ml heparin or 100ng/ml IGF1 for 10 minutes before being promptly lysed for protein. AKT activation was assayed through probing for phosphorylation at serine residue 473 on a Western blot.

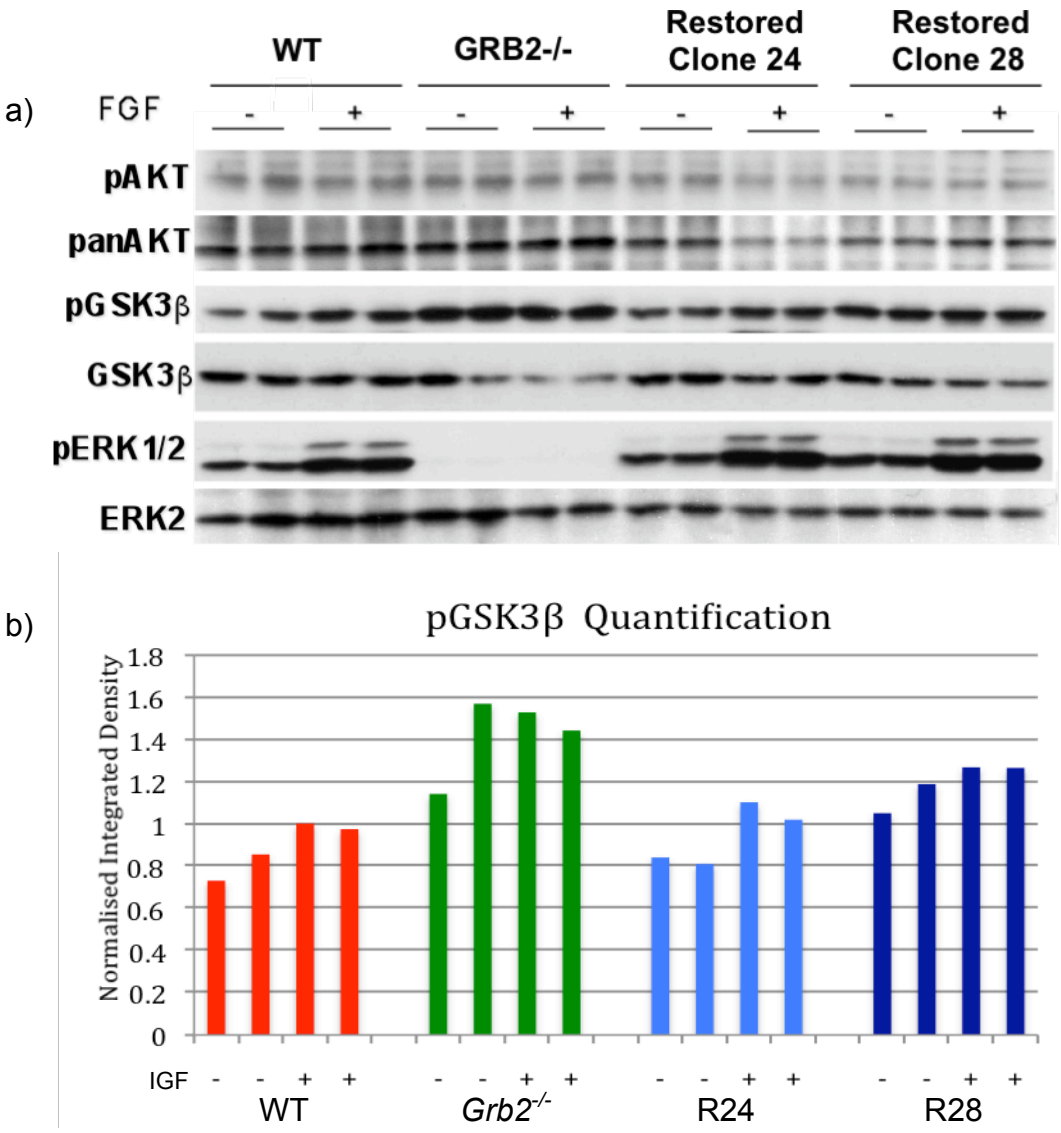


Figure 4.3 a) Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored cell lines were starved of LIF and serum for 3 hours and induced with 25ng/ml FGF2 and 1μg/ml heparin for 10 minutes before lysis and Western analysis. FGF2 did not significantly induce activation of AKT in ES cells. Some upregulation of GSK3β was observed. ERK1/2 activation was clear in all *Grb2* expressing lines. **b)** Quantification of pGSK3β. The integrated density of pGSK3β signal was measured using Image J image analysis software and normalised to the integrated density of the corresponding GSK3β signal in the blot presented above.

Unexpectedly no increase in phosphorylation of AKT was detected in response to FGF2 at 10 minutes by any of the cell lines used (Fig 4.3a).

GSK3β is phosphorylated at serine residue 9 by active AKT (Cross *et al*., 1995). There is some evidence of a small increase in GSK3β phosphorylation in response to FGF2 at this time point (Fig4.3b). This indicates that there is an increase in AKT activity in response to FGF2 which is undetectable using a method reliant on AKT phosphorylation.

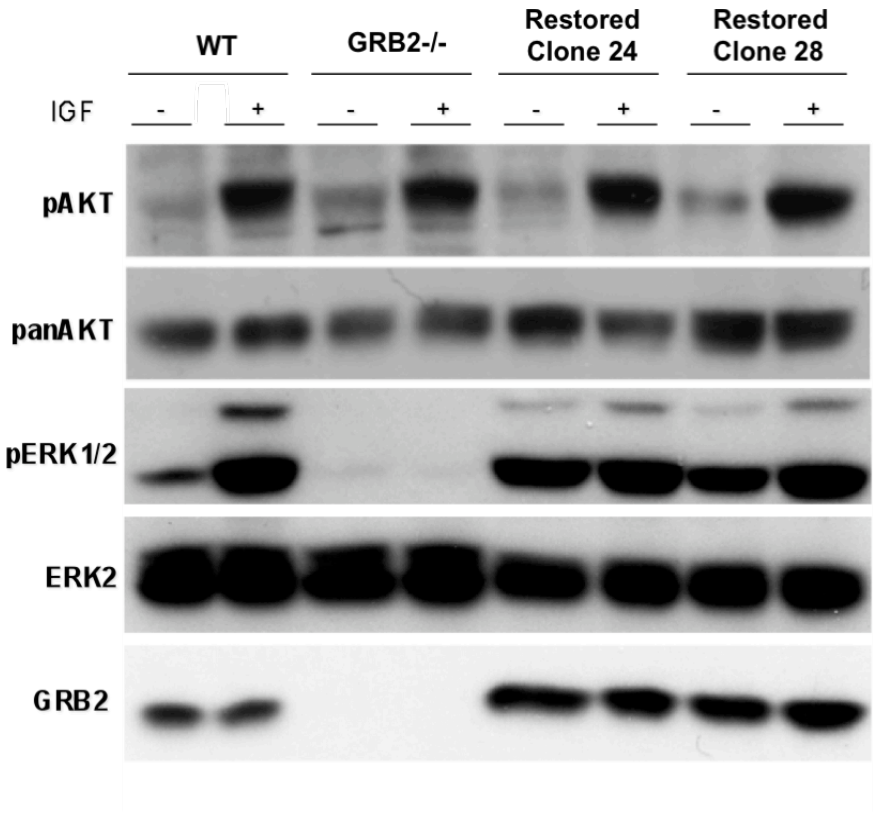


Figure 4.4 Wild type, *Grb2*$^{-/-}$ and restored cell lines were starved of LIF and serum for 3 hours and induced with 100ng IGF1 for 10 minutes before lysis and Western analysis. IGF1 induced phosphorylation of AKT in ES cells. ERK1/2 activation is clear in all *Grb2* expressing lines.

IGF1 induction confirmed activation of AKT was possible in all four ES cell lines (Fig 4.4). *Grb2*$^{-/-}$ ES cells did not exhibit any significant difference in AKT activation compared to the wild type or *Grb2* restored lines. These results indicate AKT signalling is intact in *Grb2*$^{-/-}$ ES cells.

In order to establish that the growth factors were active and FGF-mediated phosphorylation of AKT can be detected, 3T3 fibroblast cells were plated overnight, washed and starved of serum for 3 hours to reduce background signalling. After this time, cells were exposed to either 25ng/ml FGF2 and 1μg/ml heparin or 100ng/ml IGF1 for 10 minutes before being lysed for protein. AKT activation was assayed through probing for phosphorylation at serine residue 473 on a Western blot. Fig 4.5 shows clear activation of AKT in response to FGF2 and IGF1.

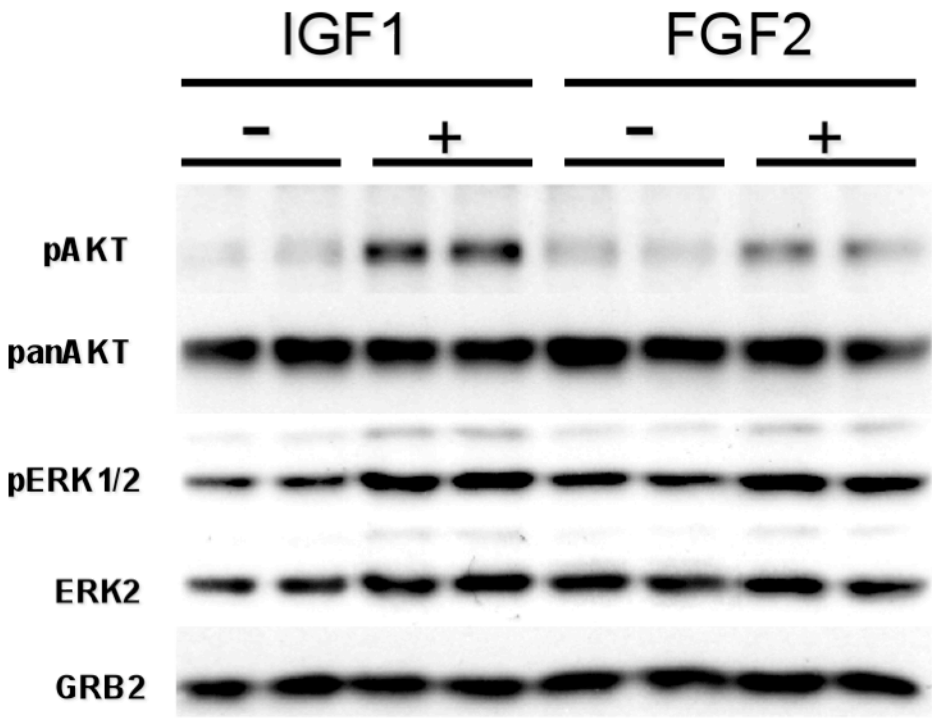


Figure 4.5 NIH 3T3 fibroblast cells were starved of serum for 3 hours and induced with either 25ng/ml FGF2 and 1μg/ml heparin or 100ng/ml IGF1 for 10 minutes before lysis and Western analysis. FGF2 and IGF1 induced phosphorylation of AKT and ERK1/2.

4.2.2. ERK Signalling is Disrupted in *Grb2*$^{-/-}$ ES Cells

The MAPK pathway is a major pathway that connects extracellular growth factor signals to their intracellular targets. Many extracellular signals have been shown to activate this pathway such as integrins (Schlaepfer *et al*., 1994), G protein coupled receptors (Buist *et al*., 1998) and growth factors (Lenormand *et al*., 1998) including those signalling through receptor tyrosine kinases (RTKs) (Reviewed in McKay and Morrison, 2007). Activation of the MAPK pathway is known to regulate many essential cellular processes for example apoptosis, growth and differentiation.

Grb2 is a key molecule in the MAPK pathway. Its major role links extracellular phosphorylation signals to Son-of-Sevenless (SOS), which in turn activates Ras GTPase. Cheng *et al*. (1998) described a fusion protein consisting of the N terminal domain of SOS fused to a *Grb2* SH2 domain. Expression of this protein bypassed the requirement for *Grb2* in ES cells and restored wild type function, indicating pathways downstream of SOS are key functions of *Grb2* in ES cells.

To investigate MAPK signalling in *Grb2*$^{-/-}$ ES cells, the levels of phosphorylated ERK were estimated in response to FGF2 and IGF1 by reprobing the Western blots constructed in the experiments described in section 4.2.1.

Figures 4.3 and 4.4 show *Grb2*$^{-/-}$ ES cells exhibit no activation of ERK in response to FGF2 or IGF1. The basal level of ERK activation was also undetectable in these cells. Wild type and restored lines showed increased ERK activation from basal levels upon stimulation with FGF2 and IGF1.

4.2.3 ERK Phosphorylation is Not Detected in *Grb2*$^{-/-}$ ES Cells Cultured in N2B27-LB

Induction experiments described previously in section 4.2.2 highlighted disruption of the MAPK signalling pathway in *Grb2*$^{-/-}$ ES cells in response to two specific stimuli; FGF2 and IGF1 (Fig 4.3 and 4.4). To investigate the activity of MAPK signalling, ES cells growing in GMEM Complete ES Cell Media or serum-free N2B27 Complete ES Cell Media (N2B27-LB) were lysed for protein and levels of activated ERK deduced by Western blot. Higher levels of activated ERK were present in serum-based media in all four cell lines. Levels of phosphorylated ERK were lower in N2B27-LB in wild type and *Grb2* restored lines and undetectable in *Grb2*$^{-/-}$ lines.

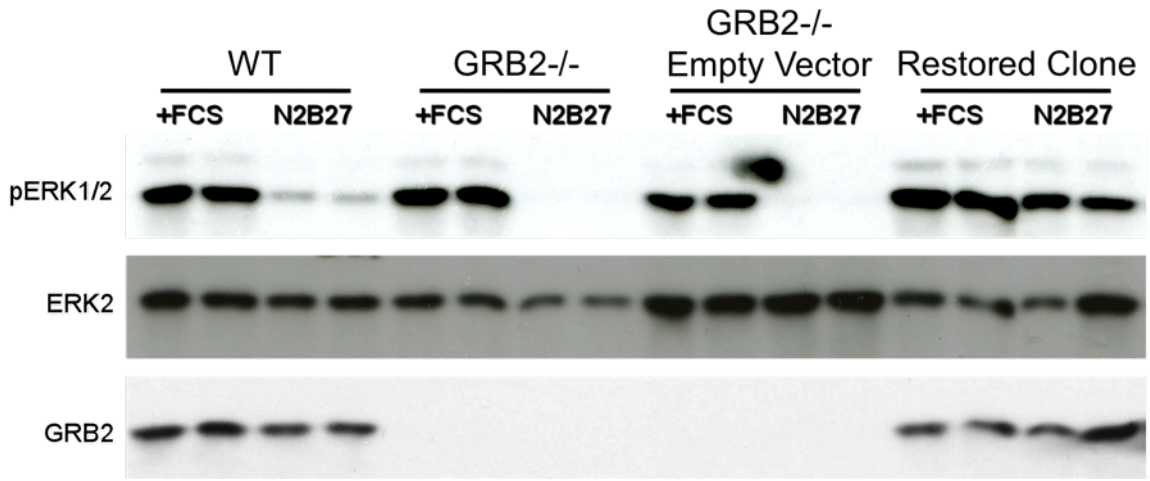


Figure 4.6 *Grb2*$^{-/-}$ ES cells exhibited no activation of ERK1/2 in N2B27. Wild type, *Grb2*$^{-/-}$, an empty vector-transfected *Grb2*$^{-/-}$ and a *Grb2* restored cell line were cultured in GMEM+FCS or N2B27-LB overnight. Protein lysates were Western analysed to show pERK signalling status.

This result indicates *Grb2*$^{-/-}$ ES cells had a deficit in ERK signalling in N2B27-LB. The level of phosphorylated ERK observed in *Grb2*$^{-/-}$ cells cultured in serum-based media appeared similar to wild type, suggesting activated ERK may be the key signal for efficient growth of ES cells.

4.2.4 Restoration of ERK signalling

ERK phosphorylation is not detected in *Grb2*$^{-/-}$ ES cells in N2B27-LB, a condition in which *Grb2*$^{-/-}$ ES cells grow poorly. Accordingly, attempts were made to restore this signal to ascertain whether ERK is the downstream effector of *Grb2* required for efficient growth in N2B27-LB.

4.2.4.1 Activation of ERK by PMA

To investigate whether MAPK/ERK activation is required for efficient growth in N2B27-LB, ERK was activated using phorbol 12-myristate 13-acetate (PMA). PMA is a phorbol diester known to mimic diacylglycerol, a ligand and activator of protein kinase C (PKC) (Verin *et al*., 2000). In order to ascertain the effect of PMA on ERK signalling in ES cells, an induction experiment replicating previous inductions was set up utilising 1000u/ml LIF or 100μM PMA as the stimulant.

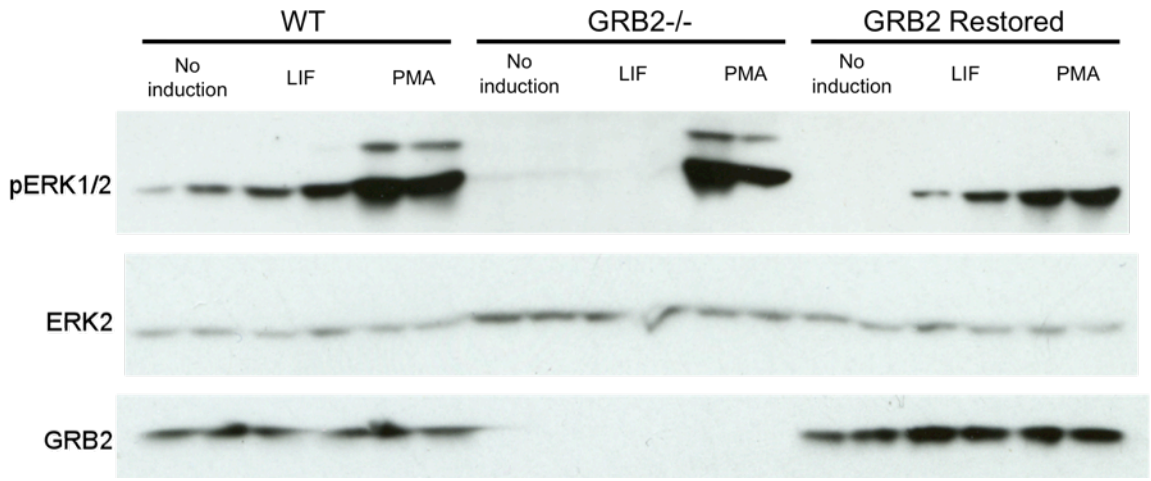


Figure 4.7 PMA induces ERK1/2 activation in *Grb2*$^{-/-}$ ES cells. Wild type, *Grb2*$^{-/-}$ and a *Grb2* restored cell lines were starved of LIF and serum for 3 hours and induced with 1000uLIF or 100nM PMA for 10 minutes before lysis and Western analysis.

10 minutes after addition of the stimulant, an increase in ERK phosphorylation in response to LIF was observed in all *Grb2* expressing lines (Fig 4.7). *Grb2*$^{-/-}$ ES cells showed no ERK phosphorylation. However, in response to PMA, all ES cell lines exhibited strong activation of ERK. At 10

minutes induction, PMA induced higher levels of ERK activation than LIF in all lines.

PMA activates ERK via upregulation of the GTP-bound, active form of Ras (Verin *et al*., 2000), although this has not been demonstrated in ES cells. To investigate the mechanism by which PMA activates ERK, cells were induced as in previous experiments with the exception of an additional 1 hour incubation in the presence or absence of PD0325901, a specific inhibitor of MEK signalling. Fig 4.8 confirms that MEK is responsible for ERK activation via PMA.

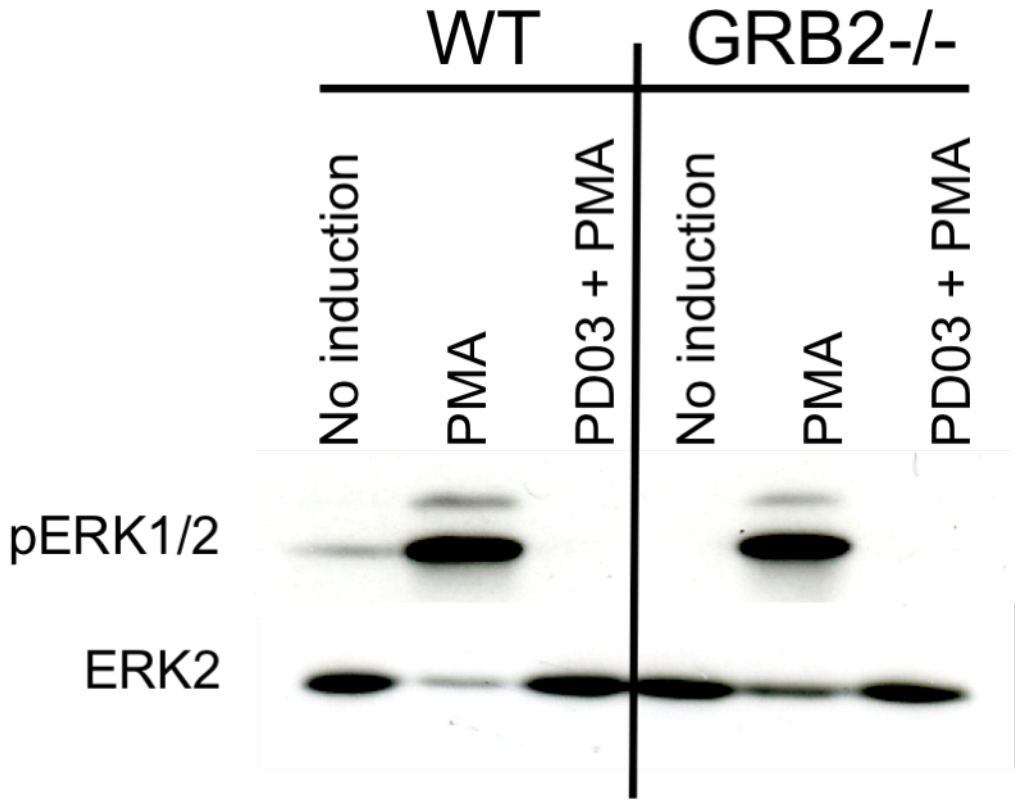


Figure 4.8 PMA activation of ERK1/2 is via MEK. Wild type and *Grb2*$^{-/-}$ ES cells were starved of LIF and serum for 3 hours, exposed to MEK inhibitor PD0325901 for 1 hour then PMA induced for 10 minutes.

These experiments show that PMA is a highly potent activator of ERK in ES cells. This activation is independent of *Grb2* but activation is MEK-

dependent, suggesting PMA activates the MAPK pathway downstream of *Grb2*. In order to investigate whether restoration of ERK signalling would improve the growth of *Grb2*$^{-/-}$ ES cells in N2B27-LB, cells were cultured in the presence of PMA or the equivalent volume of DMSO vehicle.

After 3 days, wild type and *Grb2* restored ES cells showed no change in morphology in the presence or absence of PMA and *Grb2*$^{-/-}$ ES cells retained their poor growth phenotype (Fig 4.9). A CyQuant Direct assay was used to assess the proliferation of the cells. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were cultured in replicate plates for 3 days in the presence or absence of PMA. Cell numbers were recorded at 4, 24, 48 and 72 hours and data normalised to the 4 hour measurement. This assay confirmed that there was no increase in the rate of proliferation of the four cell lines in response to PMA (Fig 4.10). Furthermore, wild type and *Grb2* restored clone 28 exhibited a significant (WT $p<0.005$; R28 $p<0.001$) decrease in cell numbers in at the 72 hour time point.

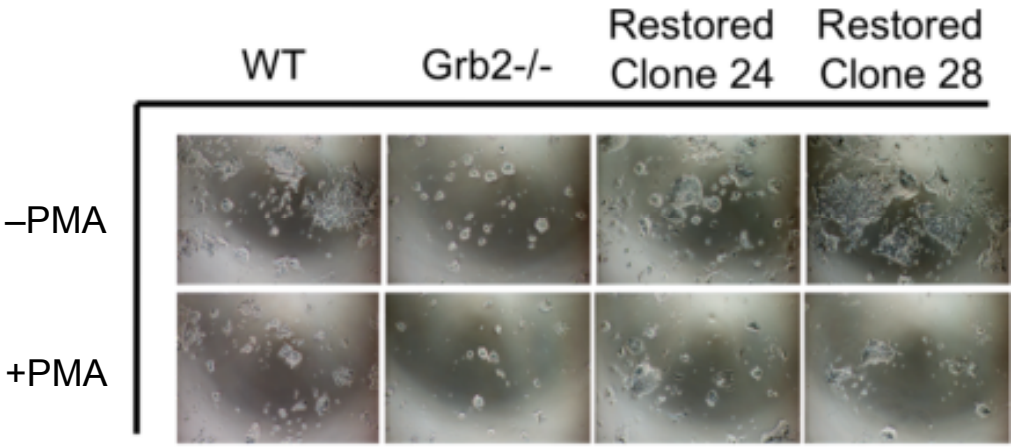


Figure 4.9 *Grb2*$^{-/-}$ ES cell morphology does not change after 3 days in N2B27-LB in the presence of 100nM PMA.

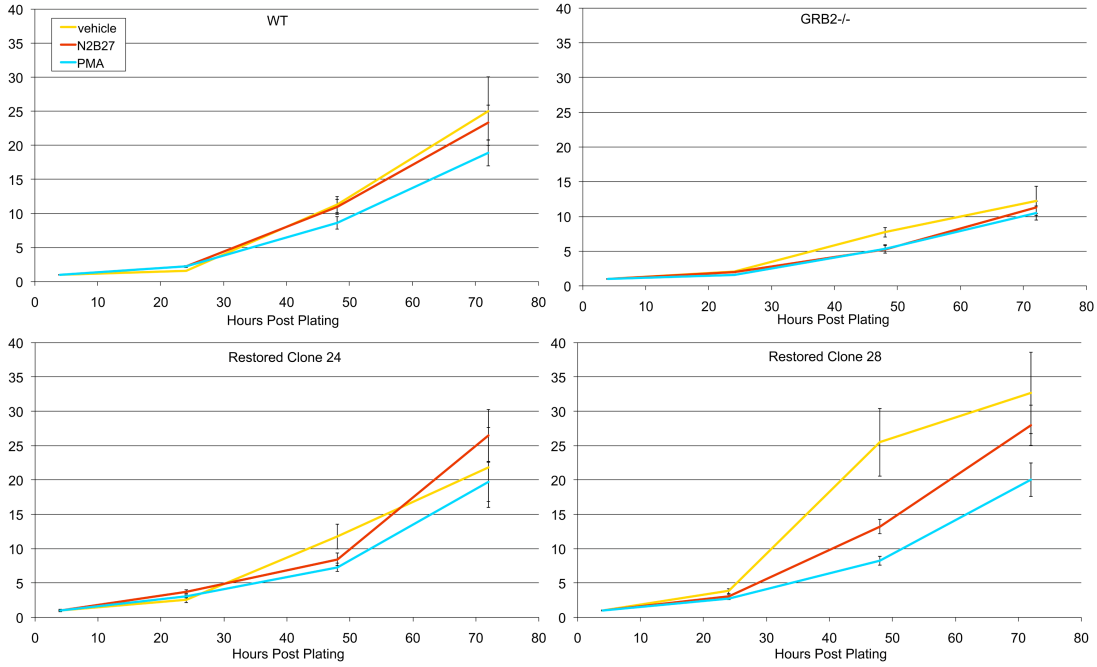


Figure 4.10 Addition of PMA did not increase the rate of growth of ES cells in N2B27-LB medium. 4x10^2 wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated per well of a 96 well plate in N2B27-LB alone and in the presence of 100nM PMA or the equivalent amount of ethanol carrier. Replicate plates were created for each time point. At 4, 24, 48 and 72 hours, cells were incubated with CyQuant Direct dye and suppressor for 1 hour before quantification using a fluorometric plate reader with a 488/520nm filter. Data was normalised to the 4-hour time point (n=12 from 2 experiments).

Although ERK activation via PMA was observed in *Grb2*$^{-/-}$ ES cells, this biochemical assay was too short-term to conclude ERK signalling was significantly restored. To investigate the duration of the ERK signal stimulated by PMA, cells were induced as in previous experiments and harvested at various time points. Wild type cells showed a peak of ERK activation at 30 minutes, which was undetectable by 8 hours (Fig 4.11). *Grb2*$^{-/-}$ cells exhibited a shorter period of ERK activation which also peaked at 30 minutes and was extinguished by 4 hours.

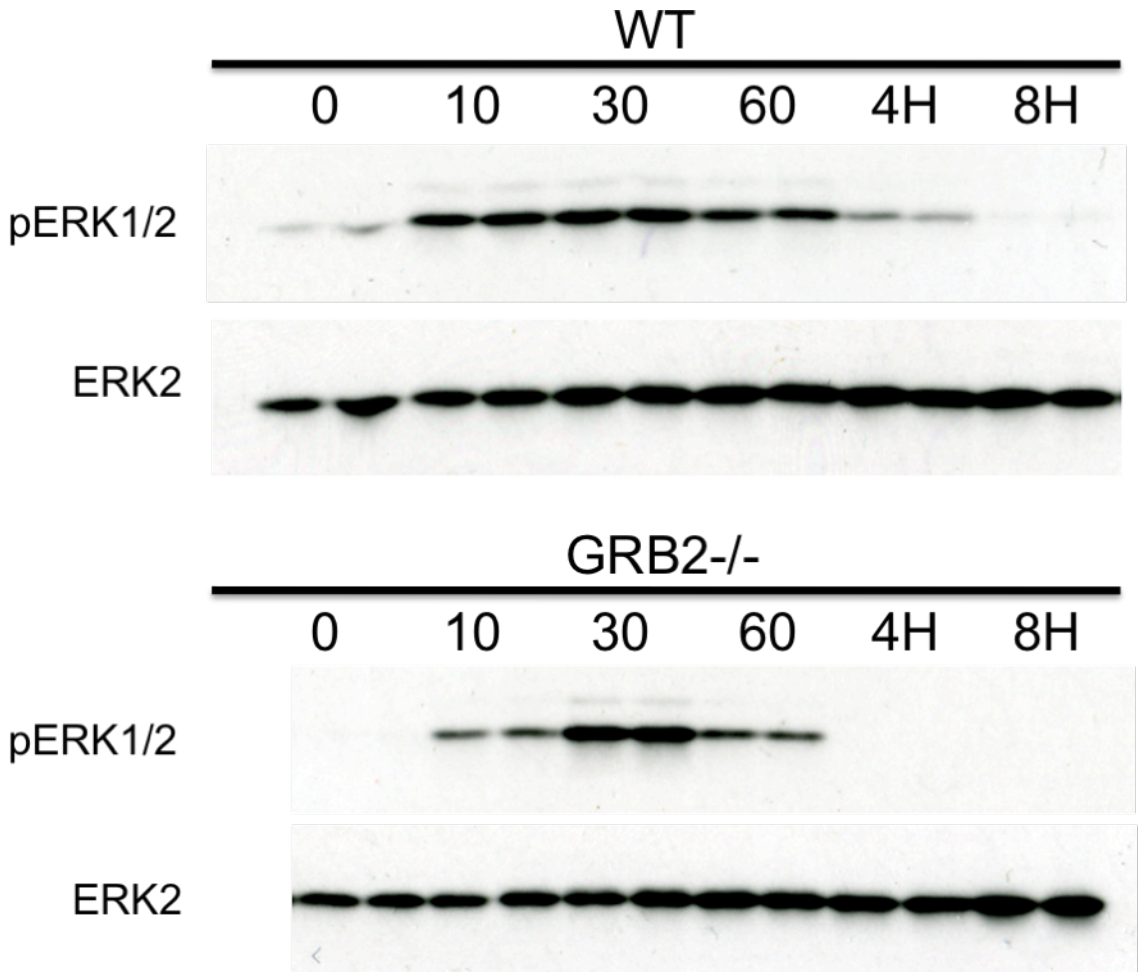


Figure 4.11 PMA induced ERK1/2 activation for over 1 hour in *Grb2*$^{-/-}$ ES cells. WT and *Grb2*$^{-/-}$ ES cells were starved of LIF and serum for 3 hours before induction with 100nM PMA. Cells were lysed at 10 minutes, 30 minutes, 1 hour, 4 hours and 8 hours and analysed by Western blot.

PMA has been verified as a *Grb2*-independent activator of ERK. When applied to N2B27-LB culture, PMA did not restore efficient growth to *Grb2*$^{-/-}$ ES cells. One explanation for this may be the brevity of the ERK signal provided by PMA, as the longevity of the ERK signal can have profound effects on the behaviour of cells. This is demonstrated clearly by PC12 cells, which respond in a different manner depending on whether ERK signalling is transient or sustained (Reviewed by Marshall, 1995). Exposure to epidermal growth factor (EGF) produces a short-lived burst of ERK activation and promotes cell proliferation, while neural growth factor (NGF) induces a more sustained activation of ERK and cells undergo differentiation.

4.2.4.2 Activation of ERK via Raf-ER

To investigate the effect of sustained ERK activation on *Grb2*$^{-/-}$ ES cells, a genetic approach was used. A plasmid containing a conditionally active kinase domain of Raf1 was used (Samuels *et al*., 1993). Amino acids 305-648 of Raf1 were shown to efficiently transform NIH 3T3 cells (Stanton, 1989) due to the truncation of the 5' regulatory domain. This constitutively active Raf1 kinase domain was fused to the HE14 oestrogen receptor sequence to enable Raf1 activation upon the addition of a 4-hydroxy-tamoxifen (4OHT) ligand. The HE14 oestrogen receptor domain contains a glycine-valine substitution at residue 400, which reduces oestrogen binding and consequently reduces unwanted activation by the oestrogen hormone (Tora *et al*., 1989).

The Raf-ER plasmid and an empty vector containing matching drug resistance were lipofected into *Grb2*$^{-/-}$ ES cells and cultured under hygromycin selection for 10 days. Hygromycin-resistant clones were expanded and tested for stable expression of a functional Raf-ER. Lines were starved of LIF and serum for three hours, exposed to 4OHT or the equivalent volume of ethanol vehicle for 2 hours or 3 days, lysed and analysed by Western blot. Detection of phosphorylated ERK in response to

4OHT rather than the ethanol vehicle was interpreted to mean Raf-ER was successfully integrated into cells and functional.

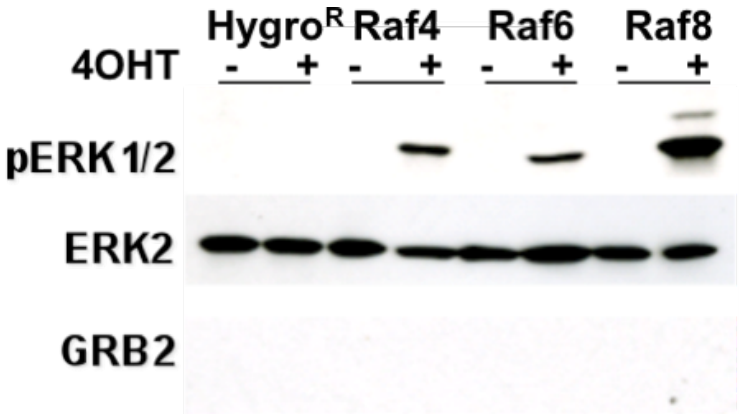


Figure 4.12 Three Raf-ER expressing, *Grb2*$^{-/-}$ clones showed an induction of ERK activation (pERK) in response to tamoxifen ligand (4OHT) and no response to the ethanol (etOH) vehicle. Empty vector transfected clones showed no ERK response to ethanol or 4OHT.

Fig 4.12 shows three Raf-ER transfected, *Grb2*$^{-/-}$ cell lines exhibiting activated ERK upon exposure to 4OHT for 2 hours. Ethanol-treated cells or cells containing an empty control vector failed to activate ERK, indicating ERK upregulation is a direct consequence of 4OHT treatment of Raf-ER expressing cells. At 3 days only one Raf-ER expressing clone exhibited detectable levels of phosphorylated ERK (Fig 4.13).

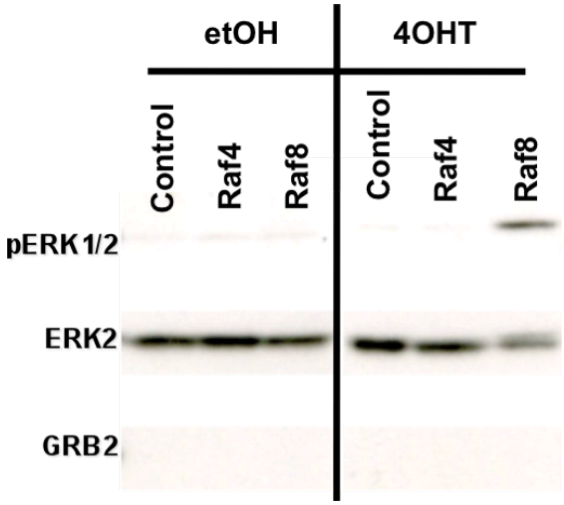


Figure 4.13 Western blot showing levels of activated ERK (pERK) after three days culture in the presence of 4OHT or ethanol vehicle.

In order to assess the effect of sustained Raf-ER activation on the signalling, morphology and identity of *Grb2*$^{-/-}$ ES cells, two *Grb2*$^{-/-}$, Raf-ER expressing ES cell lines and an empty vector-transfected control line were plated in replicate cultures in N2B27-LB with the addition of 4OHT or an equal amount of ethanol carrier. After 3 days, cells were paraformaldehyde fixed for immunohistochemical analysis.

Both Raf-ER-expressing clones performed comparably regarding morphology and Nanog intensity. Figure 4.14 shows a change in colony morphology was observed upon activation of Raf-ER. Colonies covered a greater area and lost their refractive appearance. An increase in the number of cells was also observed upon Raf-ER activation. Cells remained Nanog positive ES cells though the signal may have been less intense when Raf-ER is active.

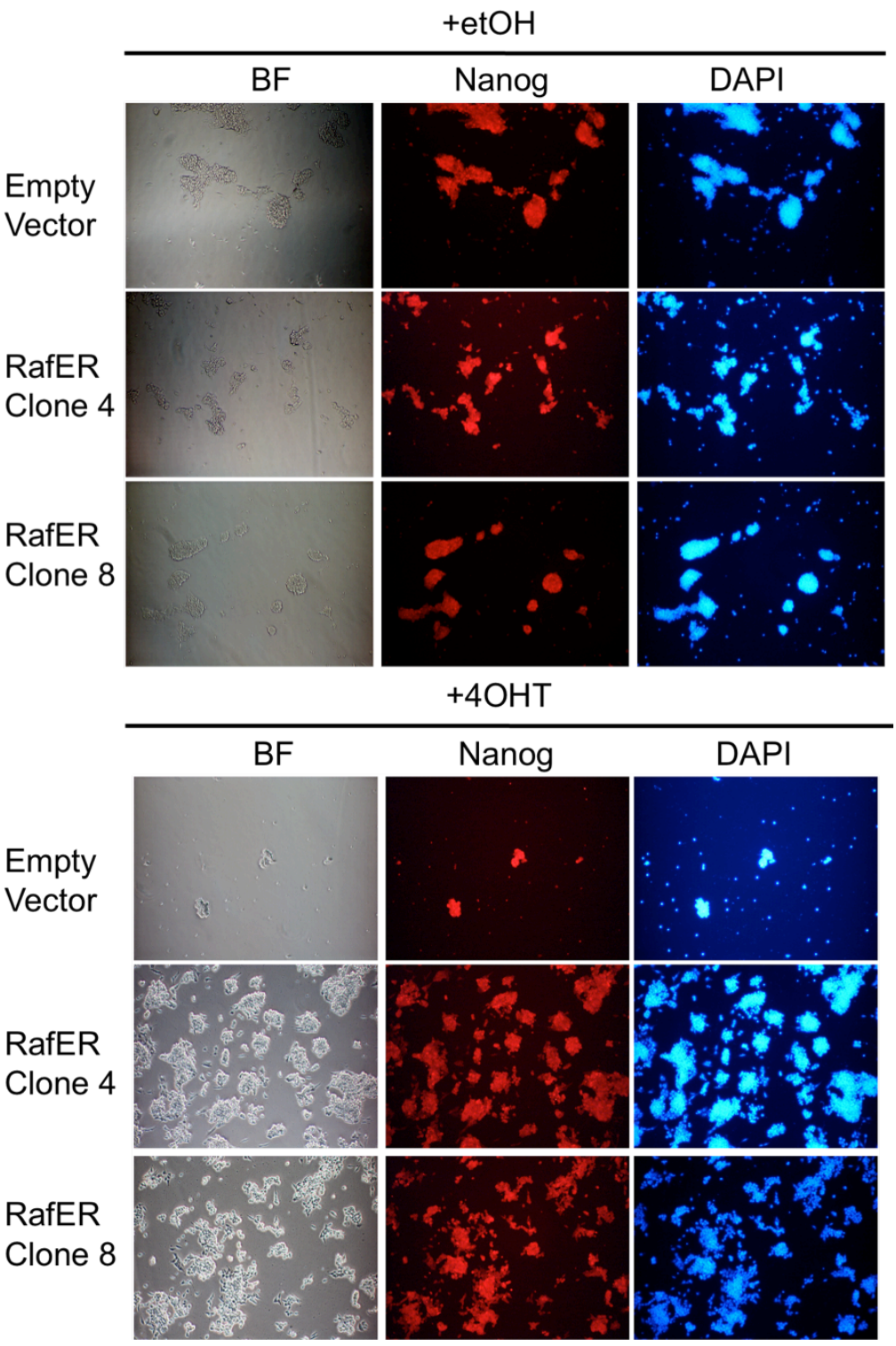


Figure 4.14 Morphology and immunohistological detection of Nanog in Raf-ER cells after growth in N2B27-LB in the presence of 4OHT or equivalent amount of ethanol carrier for three days.

To quantify the effects of conditional Raf-ER activation on *Grb2*$^{-/-}$ ES cell growth, a CyQuant Direct proliferation assay was performed (Fig 4.15). Two *Grb2*$^{-/-}$, Raf-ER expressing lines and an empty vector transfected control line were cultured in replicate plates for 3 days in the presence or absence of 4OHT or the equivalent volume of ethanol carrier. Cell numbers were recorded at 4, 24, 48 and 72 hours and data normalised to the 4 hour measurement. Proliferation of all cell lines did not increase in response to 4OHT. Furthermore, all three cell lines exhibited significantly (Empty vector $p<0.001$; Clone 4 $p<0.005$; Clone 8 $p<0.01$) reduced cell numbers at the 72 hour time point. This suggests 4OHT may have a toxic effect on ES cells in this experiment.

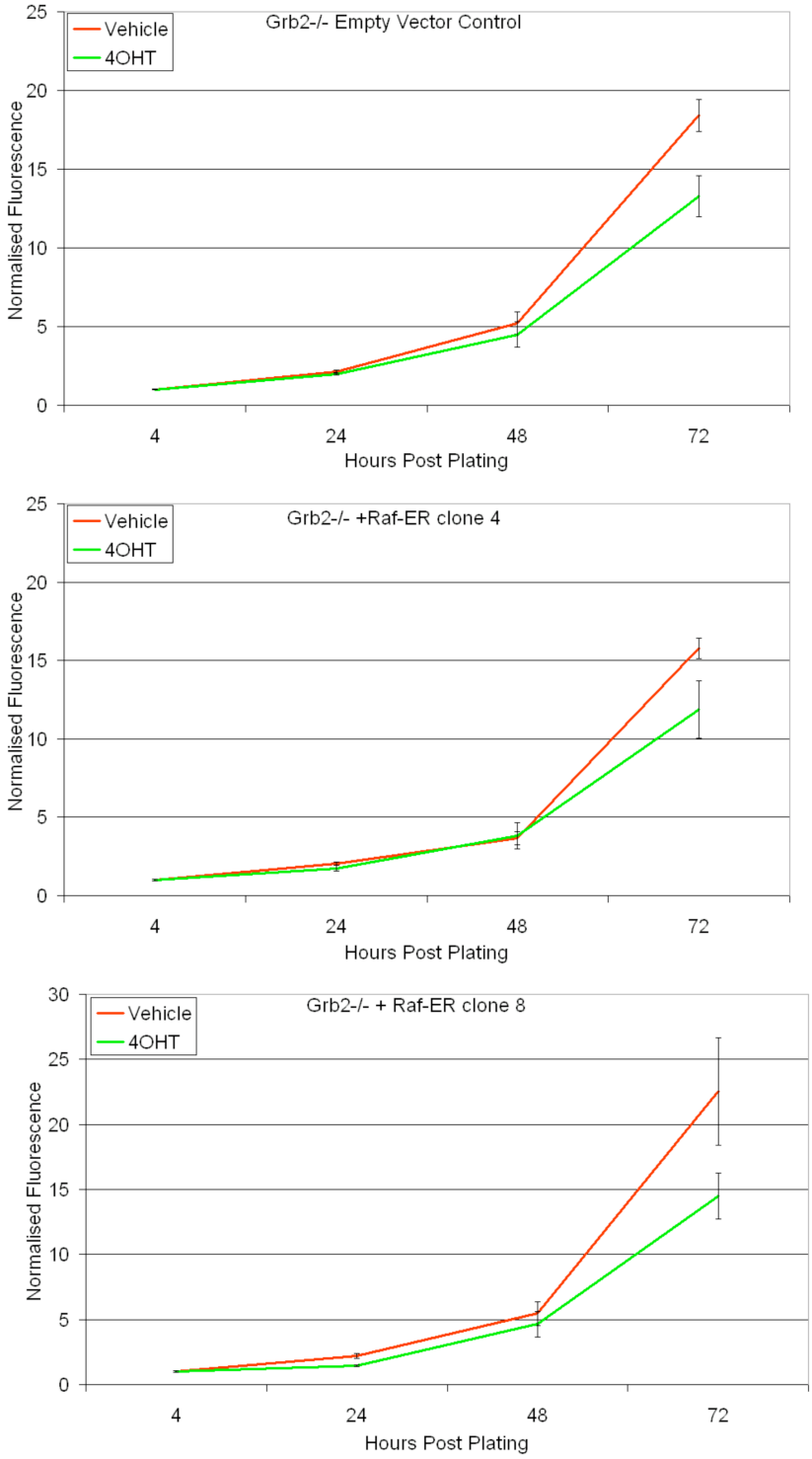


Figure 4.15 Raf-ER did not increase the rate of growth of *Grb2*$^{-/-}$ ES cells in N2B27-LB medium. 4x10^2 *Grb2*$^{-/-}$ empty vector control and *Grb2*$^{-/-}$ Raf-ER expressing cells were plated per well of a 96 well plate in the presence of 0.1μM 4OHT or the equivalent amount of ethanol carrier. Replicate plates were created for each time point. At 4, 24, 48 and 72 hours, cells were incubated with CyQuant Direct dye and suppressor for 1 hour before quantification using a fluorometric plate reader with a 488/520nm filter. Data was normalised to the 4-hour time point (n=12 from 2 experiments).

4.2.5 Addition of Small Chemical Inhibitors

So far only modulation of the MAPK pathway has been considered to improve growth of *Grb2*$^{-/-}$ ES cells in N2B27-LB. Two inhibitor compounds were considered as candidates for restoring efficient growth. Both compounds act upon specific molecular targets, which may reveal which signals are important in *Grb2*-independent growth.

4.2.5.1 ROCK inhibition does not improve growth

Rho Kinase (ROCK) is a serine-threonine kinase activated by Ras homolog gene family A (RhoA). RhoA is a ubiquitously expressed small GTPase known to regulate many pathways including actin cytoskeleton remodelling (Amano *et al*., 1997), microtubule dynamics (Kosako *et al*., 1999) and transcription factor activity (Carnac *et al*., 1998). ROCK is a downstream effector of RhoA activity. It activates LIM Kinase, which in turn phosphorylates and deactivates Cofilin, an actin-depolymerising factor. Thus, the consequence of ROCK activation is increased actin cytoskeleton stability.

Inhibition of ROCK has been associated with the inhibition of apoptosis (Koyanagi *et al*., 2008) although other studies disagree with this assertion. Sebbagh *et al*. (2001) showed ROCK inhibition by Y27632 prevented apoptotic blebbing but this did not stop the apoptotic program. This is supported by Coleman *et al*. (2001) who also demonstrated that, though ROCK is required for apoptotic body formation and the localization of DNA into these bodies, ROCK inhibition does not prevent apoptosis. This is relevant to the *Grb2*$^{-/-}$ poor growth phenotype as no increase in apoptosis was measured.

ROCK inhibition aids the passage of human embryonic stem cells (hES cells). Specifically, inhibition of ROCK enables cells to survive single cell dissociation, previously a limiting step in hES cell culture and expansion (Watanabe *et al*., 2007).

In order to assess whether ROCK inhibition improved growth of *Grb2*$^{-/-}$ ES cells in N2B27-LB, cells were cultured in N2B27-LB in the presence or absence of Y27632 ROCK inhibitor for 3 days.

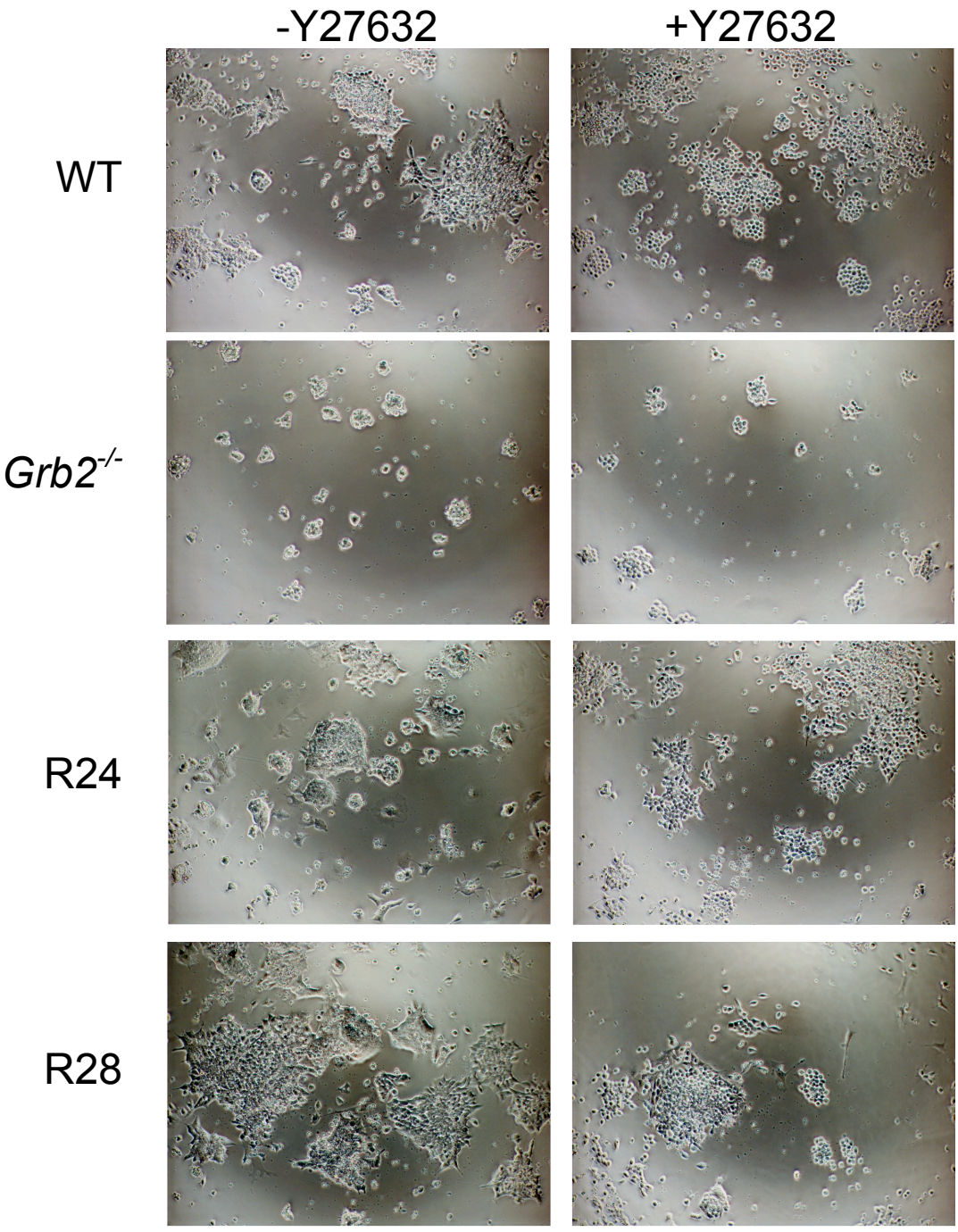


Figure 4.16 *Grb2*$^{-/-}$ ES cell morphology does not change after 3 days culture in N2B27-LB in the presence of Y27632 ROCK inhibitor.

ROCK inhibited cells adopted a spherical morphology without any cell-cell contact, whilst remaining attached to the coated issue plastic (Fig 4.16). A CyQuant Direct assay was used to assess the proliferation of the cells. Wild type, *Grb2*$^{-/-}$ and 2 *Grb2* restored ES cell lines were cultured in replicate plates for 3 days in the presence or absence of Y27632. Cell numbers were recorded at 4, 24, 48 and 72 hours and data normalised to the 4 hour measurement. This assay confirmed proliferation of all cell lines did not increase in response to ROCK inhibition (Fig 4.17).

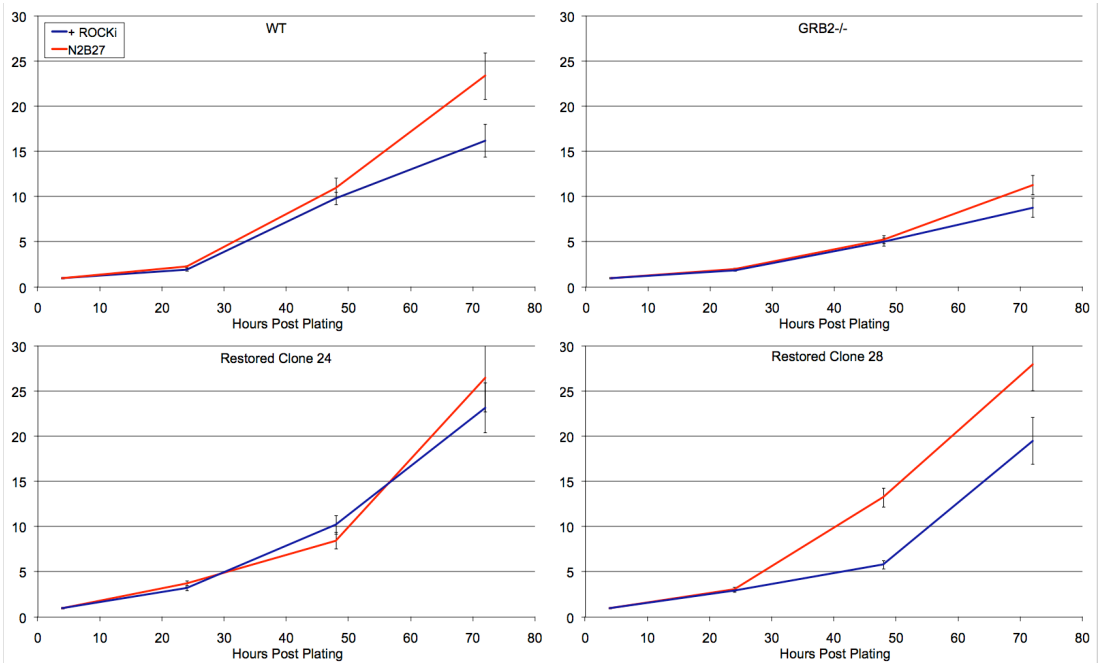


Figure 4.17 Addition of Y27632 ROCK inhibitor did not increase the rate of growth of ES cells in N2B27-LB medium. 4x10^2 wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated per well of a 96 well plate in N2B27-LB alone and in the presence or absence of 10μM ROCK inhibitor Y27632. Replicate plates were created for each time point. At 4, 24, 48 and 72 hours, cells were incubated with CyQuant Direct dye and suppressor for 1 hour before quantification using a fluorometric plate reader with a 488/520nm filter. Data was normalised to the 4-hour time point (n=12 from 2 experiments).

4.2.5.2 GSK3β Inhibition Does Not Restore Growth of *Grb2*$^{-/-}$ ES Cells

GSK3β is a serine-threonine kinase originally associated with the downregulation of glycogen synthesis (Embi *et al*., 1980) and has since been attributed to numerous cellular functions. GSK3β is constitutively active, thus it is negatively regulated by signalling pathways such as PI3K/AKT and the canonical Wnt pathway.

Sato *et al.* (2004), applied indirubin BIO (6-bromoindirubin-3'-oxime), a GSK3β inhibitor, to human and mouse ES cultures. Addition of BIO maintained undifferentiated morphological and molecular phenotypes for cells of both species in the absence of self-renewal factors. It was suggested that Wnt signalling was responsible for factor independent self-renewal although the involvement of the Wnt signalling pathway in hESCs has been strongly contested (Dravid *et al.,* 2005).

Inhibition of GSK3β has been implicated in the promotion of growth and survival of murine ES cells under MAPK inhibition. ES cells under combined inhibition of MEK and FGFR grow poorly, undergo cell cycle arrest and apoptose (Ying *et al*., 2008), although this is circumvented by the addition of CHR99021 (CHR), a GSK3β inhibitor with higher specificity than BIO (Bain *et al*., 2007). The exact mechanism by which GSK3β inhibition acts to improve growth remains unclear, though it is not thought to protect cells from entering apoptosis.

Grb2$^{-/-}$ ES cells exhibit undetectable levels of phosphorylated ERK in N2B27-LB media (Fig 4.6). It was hypothesised that *Grb2*$^{-/-}$ cells were a genetic, rather than chemical, representation of cells under MEK inhibition. In this regard, the effect of CHR on *Grb2*$^{-/-}$ cells in N2B27-LB was investigated. In order to assess whether GSK3β inhibition improved growth of *Grb2*$^{-/-}$ ES cells in N2B27-LB, cells were cultured in N2B27-LB in the presence or absence of 3μM CHR GSK3β inhibitor for 3 days.

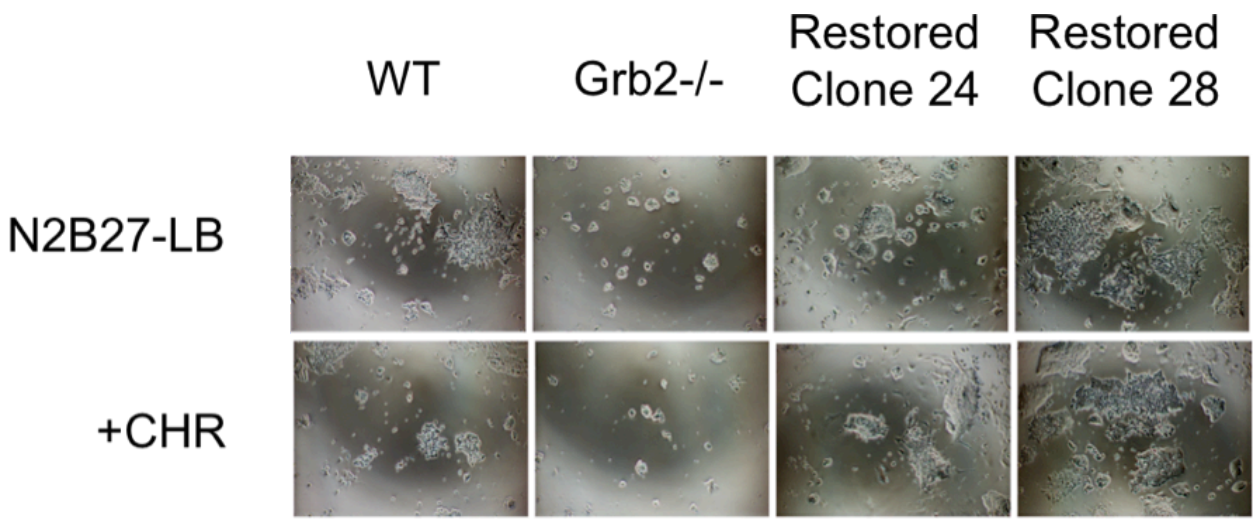


Figure 4.18 *Grb2*$^{-/-}$ ES cell morphology did not change after 3 days culture in N2B27-LB in the presence of CHR99201 GSK3β inhibitor.

After 3 days culture, the morphology of the cells did not improve (Fig 4.18). Furthermore, colonies became smaller and highly refractive, demonstrating an exaggeration of the poor growth phenotype of *Grb2*$^{-/-}$ ES cells in N2B27-LB alone.

The pluripotency of the cells was confirmed through immunohistochemical staining for ES cell marker Nanog. *Grb2*$^{-/-}$ ES cells under GSK3β inhibition stained very highly for Nanog (Fig 4.19). The levels of Nanog were quantified via image analysis. The mean fluorescence intensity of 6 images of Nanog-stained and corresponding DAPI-stained ES cells were measured using Image J image analysis software by normalising the signal to DAPI. Calculation of the intensity of Nanog signal suggested CHR-treated *Grb2*$^{-/-}$ ES cells exhibit an increase in Nanog expression (Fig 4.20).

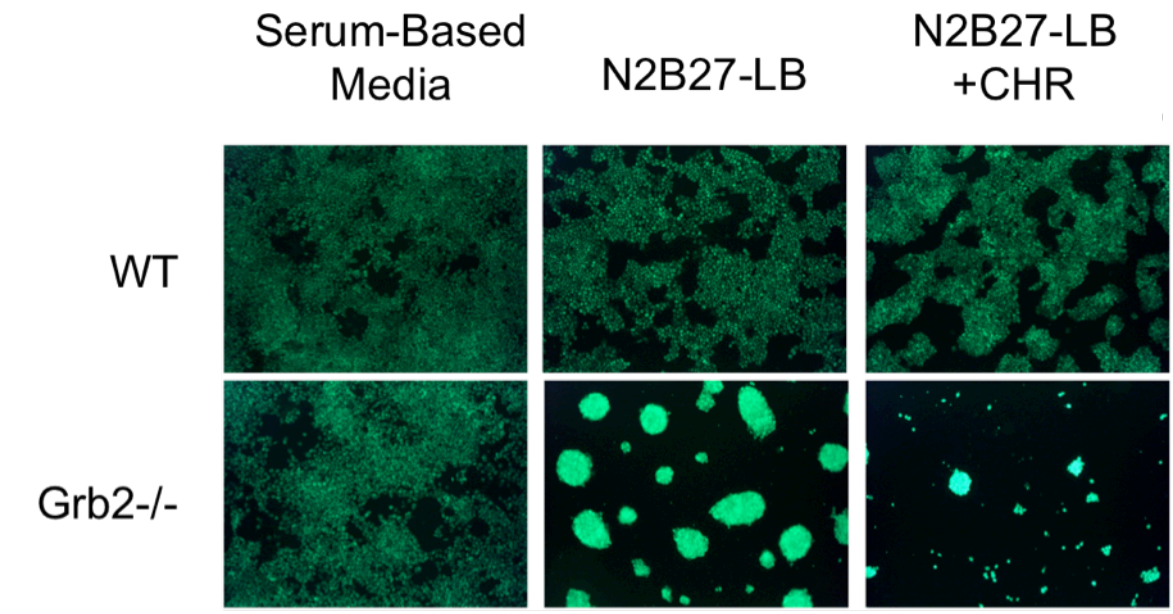


Figure 4.19 Wild type and *Grb2*$^{-/-}$ ES cells were cultured in GMEM+FCS, N2B27-LB or N2B27-LB +CHR99201 GSK3β inhibitor for 3 days. *Grb2*$^{-/-}$ ES cells stained very highly for Nanog ES cell marker.

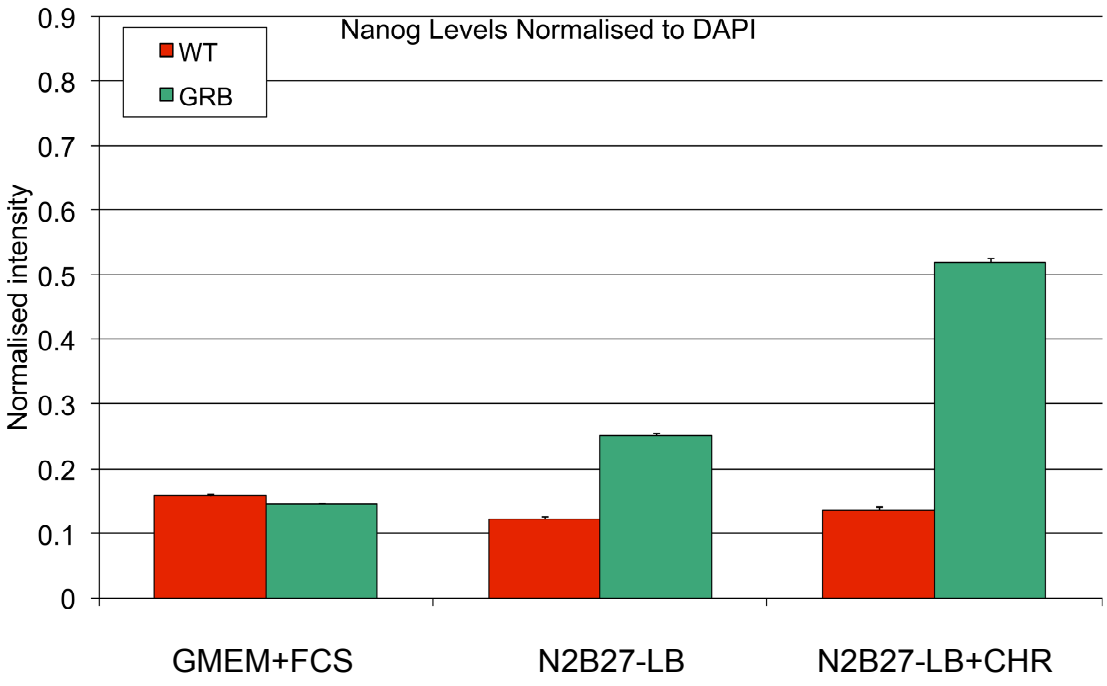


Figure 4.20 Analysis of fluorescence intensity normalised to DAPI nuclear stain. Images of 6 random fields were analysed by Image J image analysis software.

To quantify the effect CHR exerted on ES cell growth, a CyQuant Direct assay was performed. Wild type, *Grb2*$^{-/-}$ and 2 *Grb2* restored ES cell lines were cultured in replicate plates for 3 days in N2B27-LB alone or in the presence of PD03, CHR or PD03 and CHR combined. Cell numbers were recorded at 4, 24, 48 and 72 hours and data normalised to the 4 hour measurement. This assay showed CHR significantly ($P<0.001$ by student's T-test) reduced proliferation of *Grb2*$^{-/-}$ ES cells at the 72 hour time point (Fig 4.21). CHR also significantly ($p<0.01$) reduced the proliferation of wild type ES cells. However, both *Grb2* restored clones showed a significant (R24 $p<0.001$; R28 $p<0.001$) increase in cells numbers, making these data difficult to interpret. Together these data infer that GSK3β inhibition promotes self-renewal.

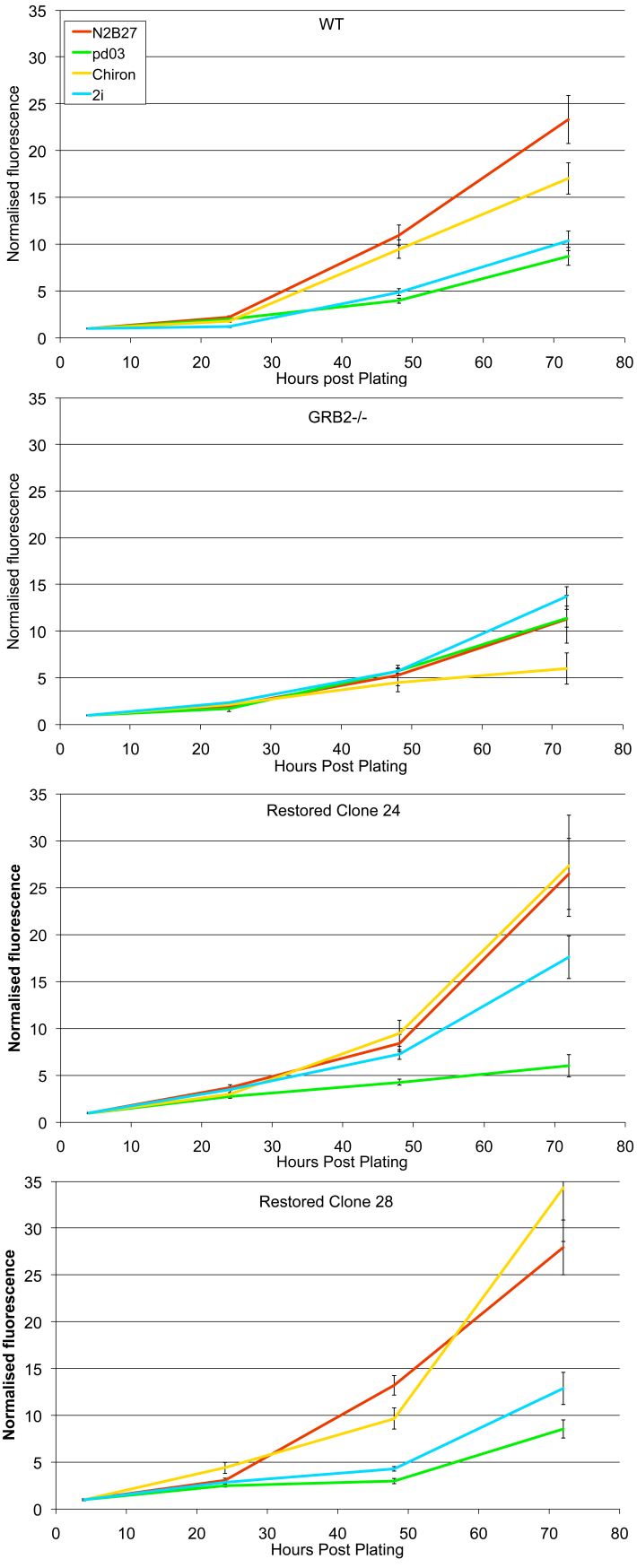


Figure 4.21 Addition of CHR99201 GSK3β inhibitor did not increase the rate of growth of *Grb2*$^{-/-}$ ES cells in N2B27-LB medium. 4x10^{2} wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated per well of a 96 well plate in N2B27-LB alone and in the presence of 3μM CHR99201, 1μM PD0325901 or both CHR990201 and PD0325901 together (2i). Replicate plates were created for each time point. At 4, 24, 48 and 72 hours, cells were incubated with CyQuant Direct dye and suppressor for 1 hour before quantification using a fluorometric plate reader with a 488/520nm filter. Data was normalised to the 4-hour time point (n=12 from 2 experiments).

4.5 Discussion

This chapter focuses on the biochemical events that contribute to efficient growth in N2B27-LB. *Grb2*$^{-/-}$ ES cells grow poorly in this media and attempts were made to understand the signals required to improve growth.

AKT signalling is intact in *Grb2*$^{-/-}$ ES cells. FGF and IGF signalling were investigated, two key factors responsible for upregulation of AKT. Phosphorylation at serine 473 of AKT in all ES cell lines was demonstrated in response to stimulation by IGF1. However, phosphorylation of AKT did not increase upon FGF2 induction in all four ES cell lines despite successful activation of the MAPK pathway in the same experiment and confirmation of the activity of the growth factors through induction of 3T3 fibroblasts. In this cell type, phosphorylation of AKT in response to FGF was demonstrated. Reprobing for phosphorylated GSK3β revealed a potential increase in activity downstream of AKT. This is supported by evidence suggesting FGF receptor and/or MEK inhibition has no effect on steady state AKT phosphorylation (Ying *et al*., 2008).

After investigating AKT signalling, the FGF and IGF induction Western blots were stripped using Restore stripping buffer and reprobed for ERK phosphorylation using pERK1/2 and then ERK2-specific antibodies. This showed FGF and IGF-induced MAPK activity in wild type and *Grb2* restored cell lines, confirming FGF induction was successful in these cell lines. Both FGF and IGF induction failed to activate ERK in *Grb2*$^{-/-}$ ES cells. Investigation of the signalling status of cells in culture revealed *Grb2*$^{-/-}$ ES cells have a deficit in ERK activation in N2B27-LB. This is supported by the reported restoration of *Grb2* dependent differentiation upon expression of a SOS-SH2 domain fusion protein (Cheng *et al*., 1998). This fusion protein is thought to restore MAPK signalling.

FGF and IGF-induced MAPK activation is disrupted in Grb2—ES cells. ERK activation was restored in *Grb2*$^{-/-}$ ES cells using two approaches: chemical induction via PMA and expression of conditionally active Raf1 (Fig4.22). Both methods were successful in providing cells with an ERK signal. Addition of PMA to N2B27-LB had little effect on growth and morphology of *Grb2*$^{-/-}$ ES cells whereas Raf-ER activation changed the appearance of the cells without restoring efficient growth.

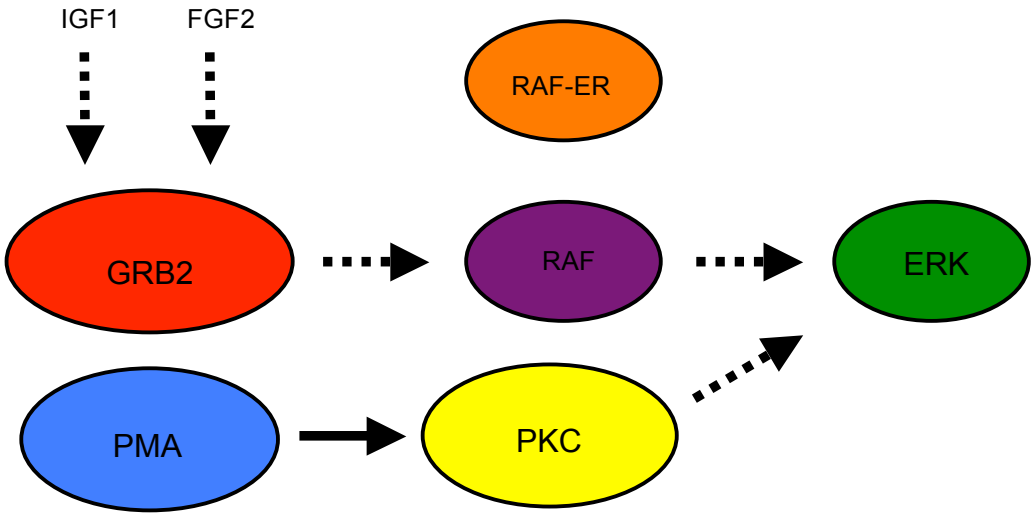


Figure 4.22 Summary of signalling events discussed in this chapter. ERK activation by IGF and FGF signalling is *Grb2* dependent. *Grb2*-independent ERK activation was achieved by two approaches. Addition of PMA induces a burst of ERK activation and induction of Raf-ER by 4OHT provides a more sustained activation of ERK.

4 hours of ERK activation via PMA induction was not sufficient to change the growth rate or morphology of *Grb2*$^{-/-}$ ES cells. This may suggest PMA induction of ERK is not equivalent to conventional GRB2-MEK-ERK signalling. PMA is regarded as a pleiotropic activator of PKC (von Lindern *et al*, 2000) due to its varying effects on the array of PKC isoforms. It is possible that ERK activation via PMA bypasses an upstream feedback loop required to spatially and temporally regulate a pathway responsible for amplifying or sustaining the ERK signal. Furthermore, chronic induction by PMA can result in down-modulation of PKC (Hofmann, 1997) which may increase apoptosis in ES cells (Quinlan *et al*., 2003).

Sustained ERK activation was achieved via the expression of a tamoxifen-inducible Raf. Activation of Raf-ER was shown to induce phosphorylation of ERK and after 3 days *Grb2*$^{-/-}$ ES cells exhibited morphology more similar to wild type.

Immunohistochemical staining revealed all cells remained Nanog positive. However, upon 4OHT exposure Raf-ER cells stained less intensely. This result is in agreement with published data showing MAPK activation represses Nanog expression (Hamazaki *et al*., 2006).

Prolonged activation of MAPK may augment differentiation of ES cells (Kunath *et al*., 2007). However, after three days of sustained Raf-ER activation cells remained Nanog positive. This may suggest Raf-ER activation is not equivalent to FGF-MEK-ERK signalling, but it is more likely that LIF and BMP4 counteract the effect of ERK activation downstream of the Raf-ER-ERK signal (Ying *et al*. 2003).

4OHT-mediated Raf-ER activation can be effective at sustained ERK activation. Activation of Raf-ER in *Grb2*$^{-/-}$ ES cells changes the adhesive properties of the colony but does not enhance growth. Interestingly, this conclusion was also made regarding the use of fibronectin or laminin as an alternative substrate in the previous chapter. In order to confirm that adhesion is mediated by MAPK signalling, the Raf-ER cells must be cultured in the presence of a MEK inhibitor such as PD03 to see if the effect of Raf-ER activation is reversed.

Despite a 10-fold reduction in comparison to the standard amount of 4OHT, this drug appeared to have a toxic effect on the cells. This is demonstrated by the reduced proliferation exhibited by empty vector-transfected cells cultured in the presence of 4OHT. This may suggest that the Raf-ER system is not suitable for use under serum-free conditions. One alternative system

that may be impart less toxicity is the FKBP-derived destabilising domain, which can be fused to a protein of interest, such as the activated Raf used in the Raf-ER system (Chu *et al.,* 2008). This protein is then ubiquitously expressed and instantly destabilised by the FKBP domain, until a Shield-1 ligand is present. There is growing evidence that this system has a low level of toxicity (Pruett-Miller *et al*., 2009).

The *Grb2*$^{-/-}$ poor growth phenotype is characterised by both slow proliferation and altered colony adhesion. Poor colony adhesion may be regulated separately from poor proliferation (Fig4.23) as results indicate pathways downstream of Raf support colony adhesion but not growth.

ROCK inhibition augments enzymatic passage of hES cells. However, ROCK inhibition did not aid efficient growth of *Grb2*$^{-/-}$ ES cells in N2B27-LB. Evidence has been put forward suggesting the increase in survival of ROCK inhibited hES cell cultures is due to changes in the adhesive properties of the cells. Addition of EGTA to cultures abrogates the effect of ROCK inhibition. This is explained by the chelation of Ca^{2+}, which disrupts the binding of calcium-dependent cell-cell adhesion molecules such as N cadherin and E cadherin (Chitaev *et al*., 1998). ROCK inhibition increases the cell adhesion properties of cells (Amano *et al*., 1997) and this facilitates the formation of aggregates in suspension. These small aggregates of hES cells are able to survive and expand into colonies, which were previously assumed to grow from a single cell.

Murine ES cells have the ability to grow from a single cell in standard ES cell media (Smith, 2001), accordingly ROCK inhibition is not required to enhance this property. *Grb2*$^{-/-}$ ES cells do not grow efficiently in N2B27-LB medium, but ROCK-mediated signalling is not responsible for the growth defect.

GSK3β inhibition mimics the effect of PI3K/AKT hyperactivation. Inhibition or deletion of components of this cell survival pathway has varying results on

ES cells. Inhibition of PI3K by LY294002 results in ES cell differentiation (Storm *et al*. 2007), therefore it is unclear what role PI3K has in ES cell growth.

Cells under MEK inhibition fail to thrive in N2B27 media (Ying *et al*., 2008) and this is rescued upon inhibition of GSK3β. It was hypothesised that *Grb2*$^{-/-}$ ES cells are equivalent to cells under MEK inhibition and GSK3β inhibition would restore efficient growth in N2B27. However, addition of GSK3β inhibitor (CHR) resulted in further reduction of growth.

There is some evidence that *Grb2*$^{-/-}$ ES cells exhibit high basal levels of GSK3β phosphorylation, although this needs to be verified by further Western blotting of more appropriate lysates, such as those presented in figure 4.6. Assaying levels of pGSK3β in wild type, *Grb2*$^{-/-}$ and *Grb2* restored ES cell lines in both GMEM+FCS and N2B27-LB media would confirm this result.

Grb2$^{-/-}$ ES cells under GSK3β inhibition also stained very highly for self-renewal marker Nanog. This may infer that GSK3β inhibition promotes self-renewal, a finding also reported by Sato *et al.* (2004).

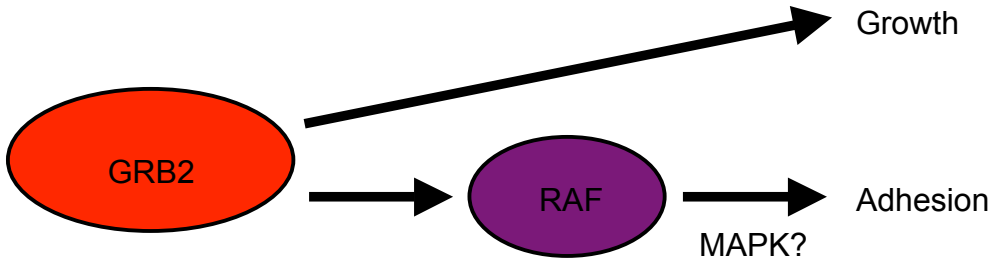


Figure 4.23 Schematic of novel findings described in this chapter. In N2B27-LB medium, *Grb2*-mediated growth and adhesion are distinct processes. *Grb2*-mediated attachment appears to be mediated by MAPK signalling.

CHAPTER 5

Grb2 is Dispensable for some Lineages of Differentiation

5.1 Introduction

Activation of the MAPK pathway is one of the earliest steps of ES cell differentiation (Kunath *et al.*, 2007; Stavridis *et al.*, 2007). Upon phosphorylation of ERK, naïve ES cells enter a reversible state in which they are receptive to differentiative signals.

Grb2$^{-/-}$ ES cells have been reported to have a limited capacity to differentiate due to abrogated GRB2-SOS-Ras-MAPK signalling (Cheng *et al.*, 1998). The previous chapter demonstrated *Grb2*$^{-/-}$ ES cells cultured in N2B27-LB exhibit undetectable levels of ERK phosphorylation. However, in the presence of serum, ERK phosphorylation was detected (fig 4.6). This suggests that one or more components of serum activate the MAPK pathway independent of Grn2.

These data raise a question: If the MAPK pathway is activated in *Grb2*$^{-/-}$ cells, what potential do they have to differentiate?

5.2 *Grb2*-Independent Differentiation

5.2.1 *Grb2*$^{-/-}$ ES cells Produce Alkaline Phosphatase Negative Cells upon Withdrawal of LIF.

Initially, to investigate the differentiation capacity of *Grb2*$^{-/-}$ ES cells, wild type and *Grb2*$^{-/-}$ ES cells were cultured in the presence or absence of LIF. After 5 days, cells were fixed and assayed for alkaline phosphatase (AP) activity. Alkaline phosphatase is a marker of pluripotent ES cells and a reduction in alkaline phosphatase activity is an indicator of ES cell differentiation. Figure 5.1 shows pink, AP positive cells were maintained in the presence of LIF for both wild type and *Grb2*$^{-/-}$ lines. The intensity of AP staining is not as strong as other AP staining presented in earlier experiments (e.g. Fig3.6) and this

was attributed to the length of time the cells were dried and stored before images were taken. As expected, LIF withdrawal resulted in spontaneous differentiation of wild type cells, demonstrated by the presence of unstained, AP negative cells. *Grb2*$^{-/-}$ ES cell also produced AP negative cells, indicating differentiation had occurred. Both cell lines differentiated to produce flat cells with some persisting ES cell colonies.

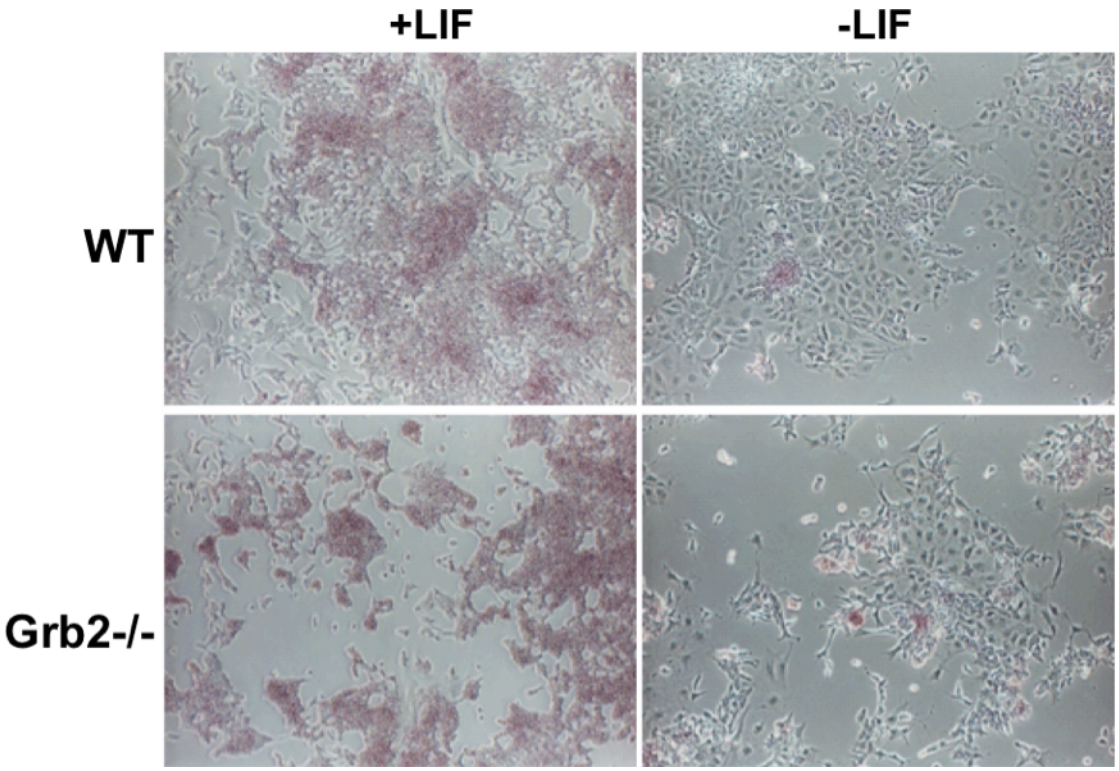


Figure 5.1 *Grb2*$^{-/-}$ ES cells spontaneously differentiate upon withdrawal of LIF in a monolayer culture. Wild type and *Grb2*$^{-/-}$ ES cells were plated at low density and cultured for 5 days in the presence or absence of LIF. After 5 days cells were fixed and stained for alkaline phosphatase activity.

5.2.2 *Grb2*$^{-/-}$ ES Cells Undergo Differentiation upon Embryoid Body Formation.

EC and ES cells form aggregates, termed embryoid bodies (EBs), when cultured in suspension. These aggregates can differentiate into all three germ layer lineages; endoderm, ectoderm and mesoderm. It was thought this differentiation happened in a disorganised manner (Martin *et al.*, 1977), however, ten Berge *et al.* (2008) showed EBs produced by ES cell

aggregation can establish anteroposterior polarity and primitive streak-like structures that mimic early embryonic patterning.

This patterning was dependent on localised Wnt signalling, demonstrating the role of morphogen gradients in imparting positional information to the developing embryo. Wnt3a, BMP4 and nodal signals were implicated in the initiation of pattern formation in embryoid bodies. Addition of Wnt3 ligand to EB cultures causes most cells to differentiate into the mesodermal lineage, whereas inhibition of this pathway results in largely ectodermal differentiation.

In vivo, the primitive streak and anteroposterior polarity are regulated by the visceral endoderm and extraembryonic ectoderm (reviewed in Tam and Loebel, 2007). Visceral endoderm produces Nodal and Wnt agonists, promoting anterior patterning and ectodermal differentiation (Glinka *et al.*, 1996; Yamamoto *et al.*, 2004).

5.2.2.1 *Grb2*$^{-/-}$ ES Cells have a Capacity to Form Advanced Cell Types, with Some Limitation.

In order to generate EBs, wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were cultured in suspension in the absence of LIF for 4 or 7 days. Grb2-/- ES cells readily formed EBs which may have been smaller than those formed by wild type or *Grb2* restored ES cells but this was not quantified. After 4 or 7 days culture in suspension, approximately 20 aggregates per cell line were plated in all 6 wells of a gelatin-coated 6-well-plate. Seven days after plating the number of beating areas of tissue were counted. One well of *Grb2*$^{-/-}$ plated EBs was fixed and immunostained for the neural markers *Nestin* and *β-III-Tubulin* after suspected neural differentiation was observed.

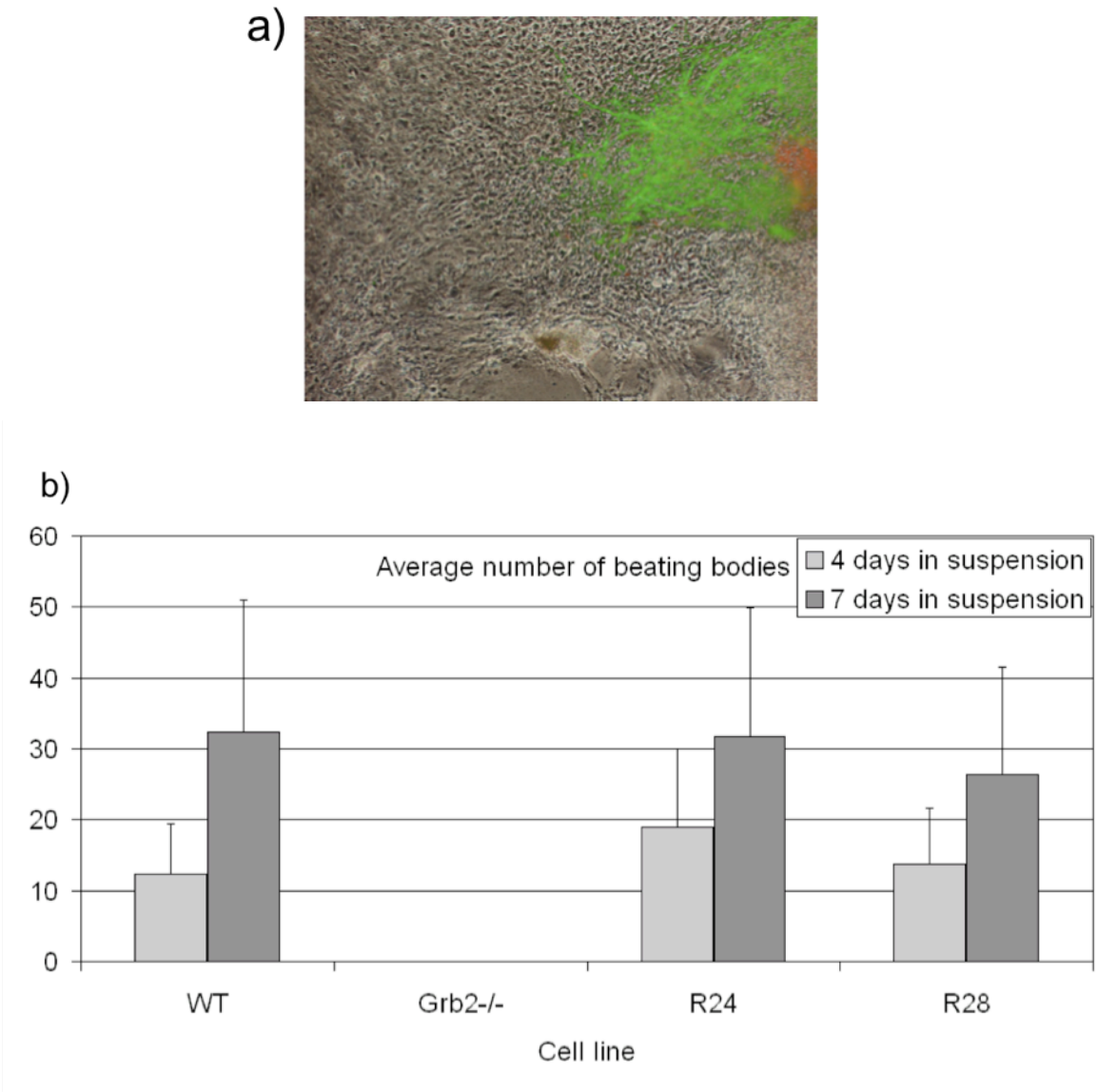


Figure 5.2 a) *Grb2*$^{-/-}$ EBs produce *β-III-tubulin* positive neurons. *Grb2*$^{-/-}$ ES cells were cultured in suspension for 7 days and the resulting EBs plated on gelatin for 7 days. After this time, cells were fixed and immunostained for neural markers *Nestin* (Red) and *β-III-tubulin* (Green). **b)** The average number of beating bodies counted after 4 or 7 day EBs plated on gelatin for 7 days. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were cultured in suspension for 4 and 7 days. Approximately 20 of the resulting EBs were plated on gelatin for 7 days. After this time, the number of beating cardiomyocyte colonies was recorded.

Figure 5.2a shows *Nestin* positive and *β-III-tubulin* positive cells. *β-III-tubulin* positive cells form a complex network of processes amongst other cells. This suggests *Grb2*$^{-/-}$ ES cells have a capacity to undergo advanced neural differentiation.

Figure 5.2b shows the average number of beating cardiomyocyte colonies observed upon plating of EBs on gelatin for 7 days following 4 and 7 days culture in suspension. Wild type and both *Grb2* restored ES cell lines produced a greater number of beating bodies after 7 days culture in suspension compared to 4 days. *Grb2*$^{-/-}$ ES cells failed to produce beating colonies in all experiments.

5.2.2.2 *Grb2*$^{-/-}$ EBs Express Markers of all Lineages Excluding Endoderm.

To investigate which germ layer lineages *Grb2*$^{-/-}$ ES cells are able to generate, wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were cultured in suspension. EBs were harvested at 2, 4, 6, 8 and 10 days culture in suspension. RNA was extracted and DNase treated to eliminate genomic DNA. cDNA was synthesised using the Invitrogen 'Superscript First-Strand cDNA Synthesis' kit for PCR analysis of gene expression.

cDNA samples were assayed for numerous markers by PCR. Figure 5.2c shows markers expressed by ES cells and EBs differentiating for up to 10 days. As a loading control, housekeeping gene *β actin* was amplified. All samples produced a single band at an equivalent intensity.

Primers were designed to amplify the full sequence of *Grb2*. In wild type cells a band of approximately 700bp is detected. *Grb2*$^{-/-}$ ES cells express a truncated, frameshifted form of the *Grb2* mRNA (Cheng *et al.*, 1998) and this was detected as a band of approximately 400bp by RT-PCR. *Grb2* restored lines express both the functional and non-functional *Grb2* mRNAs. This enables the identification of each sample as wild type, *Grb2*$^{-/-}$ or *Grb2* restored by RT-PCR.

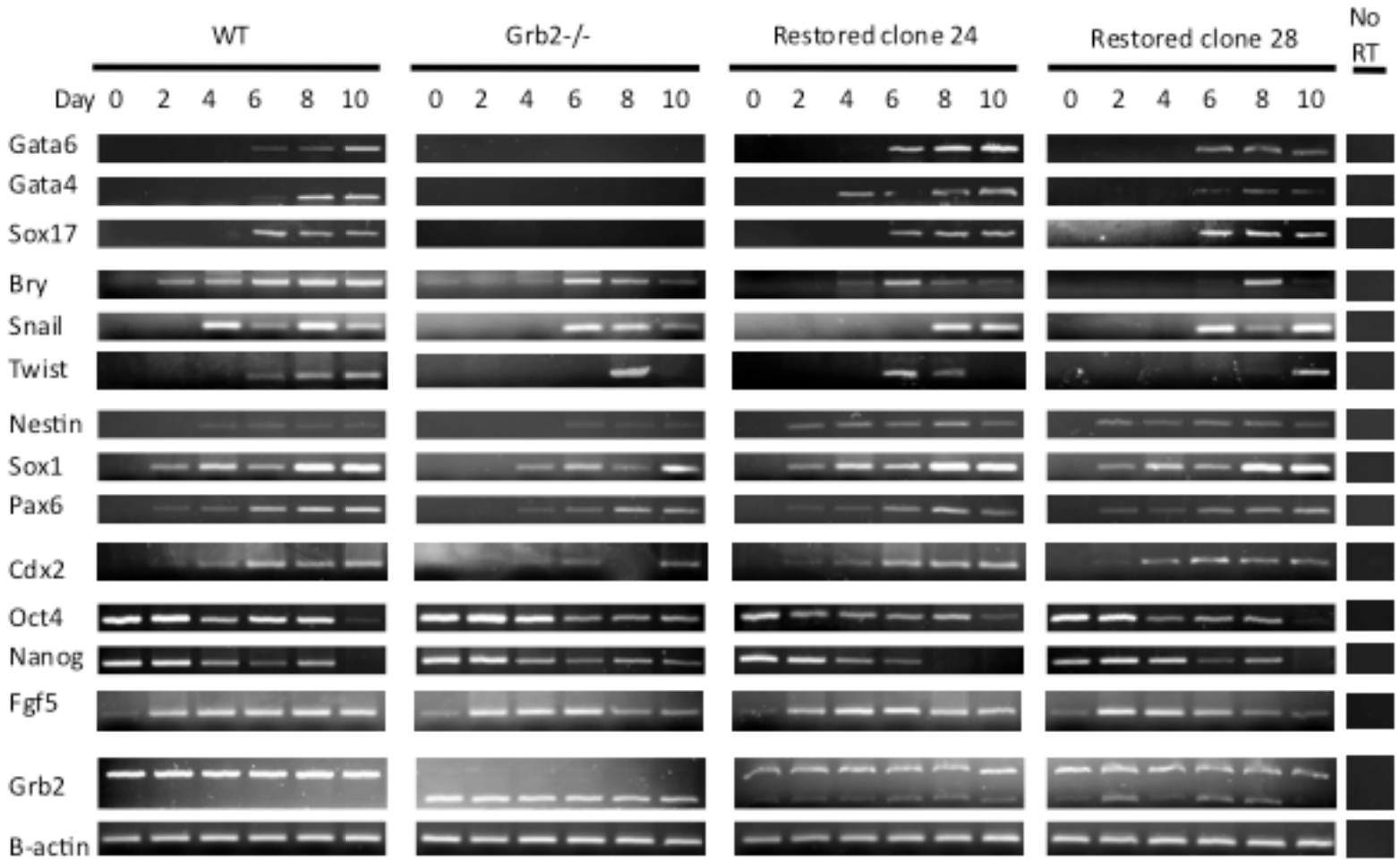


Figure 5.2c Grb2-/- EBs do not express endodermal markers. RT-PCR analysis of EB cultures at days 0-10.

ES cell markers *Oct4* and *Nanog* are highly expressed in all ES cell cultures. Both markers are detected in EB cultures and, as a general trend, appear to be downregulated over time in all cell lines. Expression in *Grb2*$^{-/-}$ EBs may be slightly more persistent, while Restored clone 24 (R24) appeared to downregulate these markers earlier.

Fgf5 is a marker of the late epiblast and early ES cell differentiation. Figure 5.2c shows weak detection of *Fgf5* in all ES cell cultures. *Fgf5* is detected in differentiated EBs, indicating all ES cell lines undergo differentiation through a late epiblast-like state.

Caudal-type homeodomain protein *Cdx2* is a transcription factor expressed by the trophectoderm lineage. Trophoblast cells form the peripheral wall of the developing blastocyst at embryonic stage E3.5 and become the placenta and membranes required for implantation and nourishment of the developing embryo. Figure 5.2c shows detection of *Cdx2* in all differentiating EB cultures, indicating all cell lines have the capacity to form trophoblast.

Ectoderm is the outermost germ layer that forms the skin, nerves and brain of the developing organism. *Nestin*, *Sox1* and *Pax6* are markers of ectodermal differentiation. *Nestin* is a type VI intermediate filament protein (Liem, 1993) considered to be a marker of multi-lineage progenitor cells (Wiese *et al.*, 2004). Sex determining region Y-box 1 (*Sox1*) is the earliest known, neuroectodermal-specific marker gene. *Sox1* expression is initially detected upon formation of the neuroectoderm. *Pax6* is part of the paired box family of transcription factors and is initially expressed in radial glial cells, which give rise to the majority of the neurons in the cerebral cortex.

Figure 5.2c shows expression of *Nestin*, *Sox1* and *Pax6* in all cell lines upon EB formation. These markers are detected later in *Grb2*$^{-/-}$ differentiating EBs compared to wild type and *Grb2* restored lines.

Cells of the mesodermal lineage form the tissues of many organs including the heart, muscles and skeleton of the developing organism. Mesoderm forms during gastrulation from epithelial cells in a process termed the epithelial to mesenchyme transition (EMT). In this process epithelial cells undergo cytoskeletal remodelling, lose polarity and cell-cell contact to adopt a migratory cell type (reviewed in Thiery, 2002). *Brachyury*, *Twist* and *Snail* are considered markers of mesodermal differentiation.

Brachyury is a T-box transcription factor, which was first described after the discovery of a mouse with a mutation in the *Brachyury* gene (Dobrovolskaïa-Zavadskaïa, 1927). *Twist1* and *Snail* are highly conserved zinc-finger transcription factors implicated in the EMT and mesodermal differentiation. Snail is initially expressed in the primitive streak at embryonic stage E7.5 and is required for the formation and maintenance of mesoderm tissues (Smith *et al.*, 1992). *Twist1* mRNA is first expressed in mesoderm outside of the primitive streak at embryonic stage E7 (Gitelman, 1997), although protein is not detectable until E8.25. Figure 5.2c shows upregulation of *Brachyury*, Snail and Twist as EB differentiation progresses in all cell lines. As expected (Rivera-Perez and Magnuson, 2005), *Brachyury* expression appeared to be downregulated by day 10 in *Grb2*$^{-/-}$ and *Grb2* restored EBs. However, wild type EBs did not appear to downregulate *Brachyury* expression. This may be explained by heterogeneous progression through the stages of differentiation or defective differentiation through the mesodermal lineage. This should be investigated further using a more quantitative method such as qPCR.

Endoderm differentiation occurs at two points in development. Extraembryonic endoderm forms at embryonic stage E3.5 in the ICM and contributes to the lining of the yolk sac. The definitive endoderm lineage forms at the primitive streak at embryonic stage E6-6.5 and goes on to produce the gastrointestinal tract, respiratory tract and urinary system of the

developing organism. *Gata4, Gata 6* and *Sox17* are markers of the endodermal lineage.

The GATA transcription factors are named after the (A/T) GATA (A/G) consensus sequence they bind. There are six members of this zinc finger binding protein family in mammals named GATA1-6. Sex determining region Y-box 17 (*Sox17*) is a member of a family of transcription factors involved in the regulation of embryonic development and in the determination of cell fate. Expression is first detected in the extraembryonic endoderm at embryonic stage E6.0 and in the definitive endoderm developing at the primitive streak at stage E7.0. Figure 5.2c shows expression of *Gata4, Gata6* and *Sox17* endodermal markers in wild type and *Grb2* restored EBs. *Grb2*$^{-/-}$ EBs do not express any of these markers, indicating a limited capacity for endodermal differentiation.

5.2.2.3 *Grb2* is Not Required for Differentiation Under Defined Conditions.

In order to investigate the potency of *Grb2*$^{-/-}$ ES cells in a defined culture system, wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated at low density in N2B27 media for 14 days. Cells were passaged at day 7. Chapters 3 and 4 describe the poor attachment of *Grb2*$^{-/-}$ ES cell colonies to gelatin in N2B27 media. To overcome this, cells were plated on laminin as well as gelatin as described in Ying *et al.* (2003). After 14 days, cells were fixed and immunostained for ES cell marker *Oct4* and neural marker β-III-tubulin.

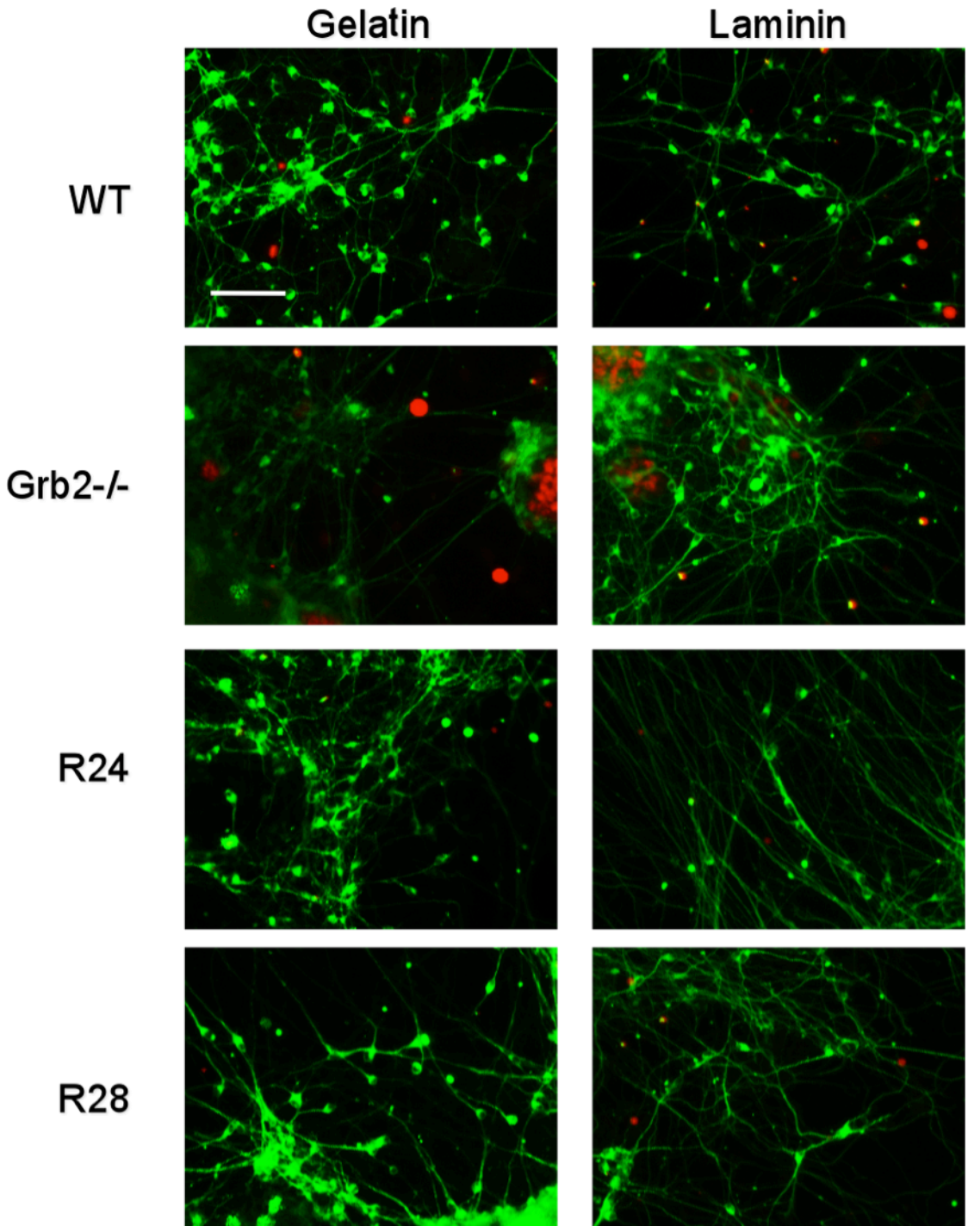


Figure 5.3 *Grb2*$^{-/-}$ ES cells differentiate in a defined culture system. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated in N2B27 on gelatin or laminin coated plates at low density for 14 days. Cells were passaged at day 7. After 14 days, cells were fixed and immunostained for ES cell marker *Oct4* (Red) and neural marker *β-III-tubulin* (Green). Scale bar – 50μm.

Figure 5.3a shows *β-III-tubulin* positive neural processes were observed in all cell lines. There was no observable difference in neural differentiation in wild type or *Grb2* restored cells when plated on gelatin or laminin. However, *Grb2*$^{-/-}$ ES cells produced a greater number and longer neural processes upon plating in laminin, suggesting laminin aids neural differentiation in the absence of *Grb2*. After 14 days, wild type and *Grb2* restored cell lines exhibited no *Oct4* positive cells. However, some *Oct4* positive ES cell colonies persisted in *Grb2*$^{-/-}$ cultures.

5.2.2.4 Neural Differentiation Occurs in the Presence of PD0325901

Chapter 4 shows undetectable levels of phosphorylated ERK in *Grb2*$^{-/-}$ ES cells in N2B27-LB. Despite this, *Grb2*$^{-/-}$ ES cells generated advanced cell types when cultured for 14 days in N2B27 alone. This suggests *Grb2*$^{-/-}$ ES cells have a capacity to differentiate independent of the MAPK pathway. To investigate this, attempts were made to inhibit ERK signalling in cells undergoing neural differentiation.

To demonstrate the efficacy of MEK inhibitor PD0325901 (PD03), wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were cultured for 24 hours in three different media: GMEM with FCS, N2B27-LB and N2B27 with 1μM PD03. After 24 hours cells were lysed and assayed for ERK phosphorylation by Western blot.

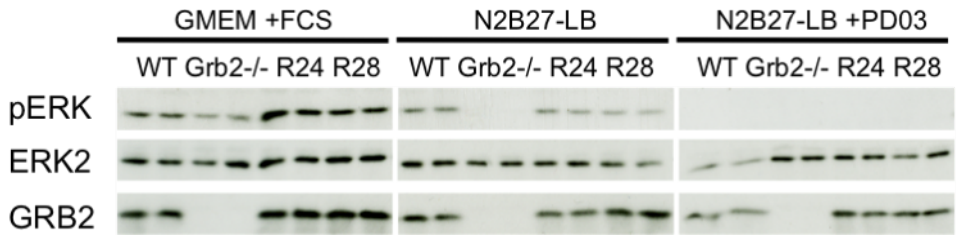


Figure 5.4 ERK signalling is inhibited by PD0325901. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were cultured for 24 hours in GMEM +FCS, N2B27-LB or N2B27-LB + 1μM PD03. Protein samples were collected and harvested for tyrosine phosphorylation of ERK1/2.

Figure 5.4 shows all cell types exhibit ERK phosphorylation in the presence of serum. Plating in N2B27-LB reduces ERK phosphorylation in all cell lines. However, no phosphorylated ERK is detected in *Grb2*$^{-/-}$ ES cells. In the presence of 1μM PD03, ERK phosphorylation was not detected in wild type, *Grb2*$^{-/-}$ or *Grb2* restored cell lines.

In order to investigate the role of ERK signalling in neural differentiation, wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated at low density in N2B27 media in the presence of 1 and 2 μM PD03 MEK inhibitor for 14 days. Cells were passaged on day 7. In addition to gelatin, a laminin substrate was utilised to aid colony attachment of *Grb2*$^{-/-}$ cells. After 14 days culture, cells were fixed and immunostained for *Oct4* and β-III-tubulin.

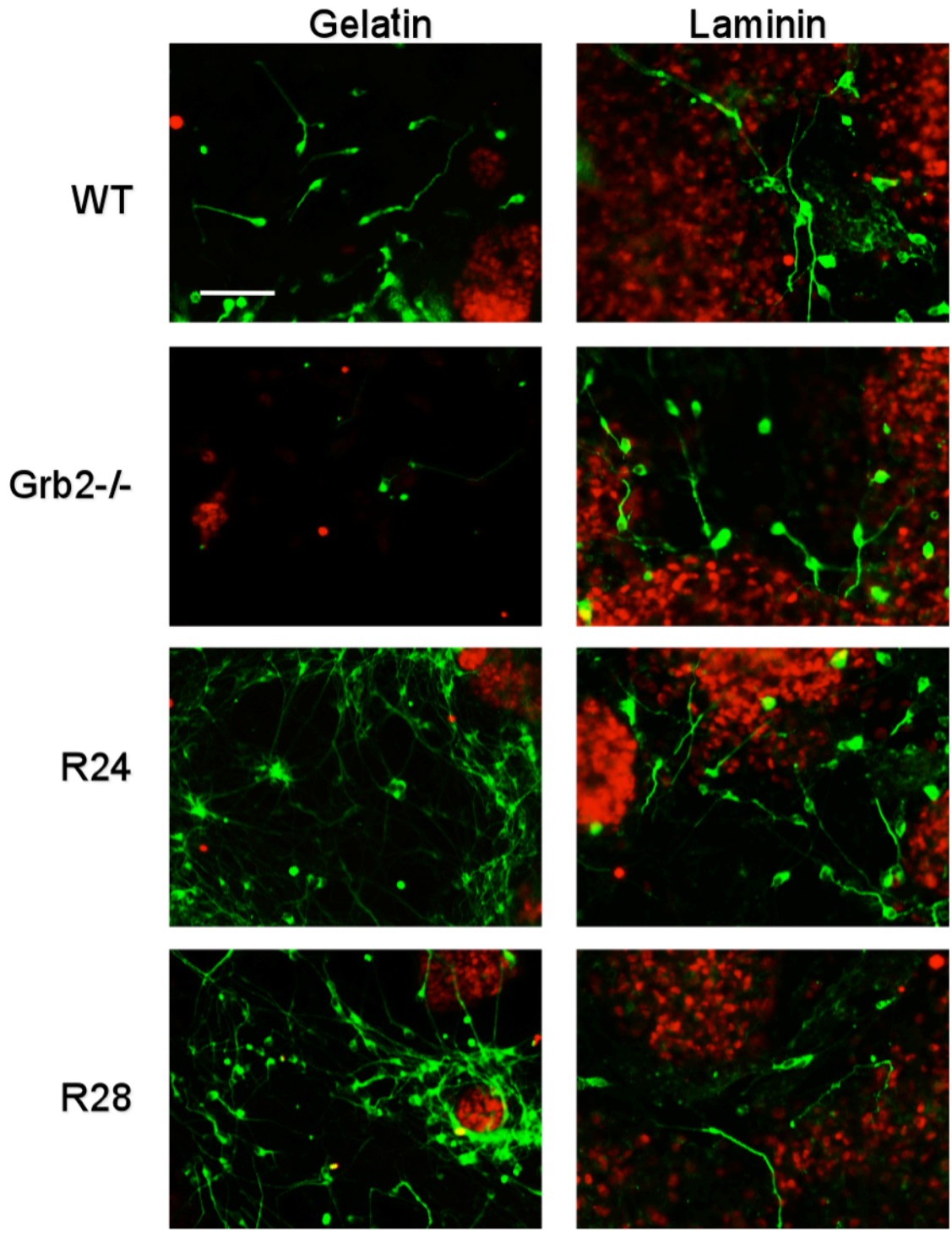


Figure 5.5 *Grb2*$^{-/-}$ ES cells differentiate in a defined culture system. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated in N2B27 + 1μM PD0325901 on gelatin and laminin coated plates at low density for 14 days. Cells were passaged at day 7. After 14 days, cells were fixed and immunostained for ES cell marker *Oct4* (Red) and neural marker *β-III-tubulin* (Green). Scale bar – 50μm.

Figure 5.5 shows neural differentiation was observed in all cell lines when cultured in the presence of 1μM PD03. On gelatin and laminin substrates, wild type and *Grb2* restored lines retained *Oct4* expressing colonies. On laminin, *Oct4* positive cells covered a larger area and fewer neural processes were observed compared to gelatin. *Grb2*$^{-/-}$ ES cells failed to expand efficiently on gelatin but some neural processes were observed. When plated on laminin, *Grb2*$^{-/-}$ ES cells exhibited an increased number of *Oct4* positive cells under PD03 MEK inhibition compared to inhibitor-free culture. Numerous *β-III-tubulin* neural processes were also observed.

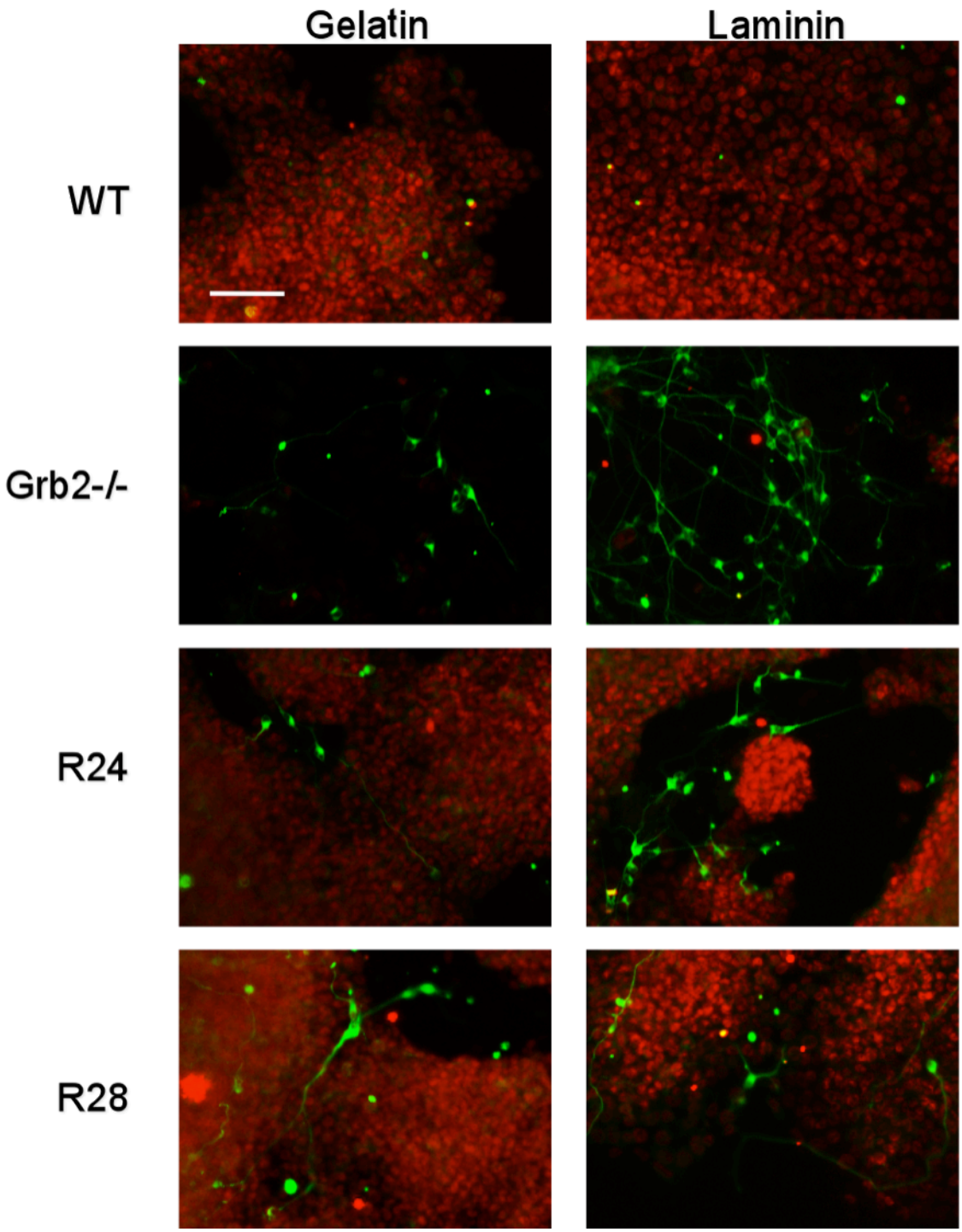


Figure 5.6 *Grb2*$^{-/-}$ ES cells differentiate in a defined culture system. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated in N2B27 +2μM PD0325901 on gelatin or laminin coated plates at low density for 14 days. Cells were passaged at day 7. After 14 days, cells were fixed and immunostained for ES cell marker *Oct4* (Red) and neural marker *β-III-tubulin* (Green). Scale bar – 50μm.

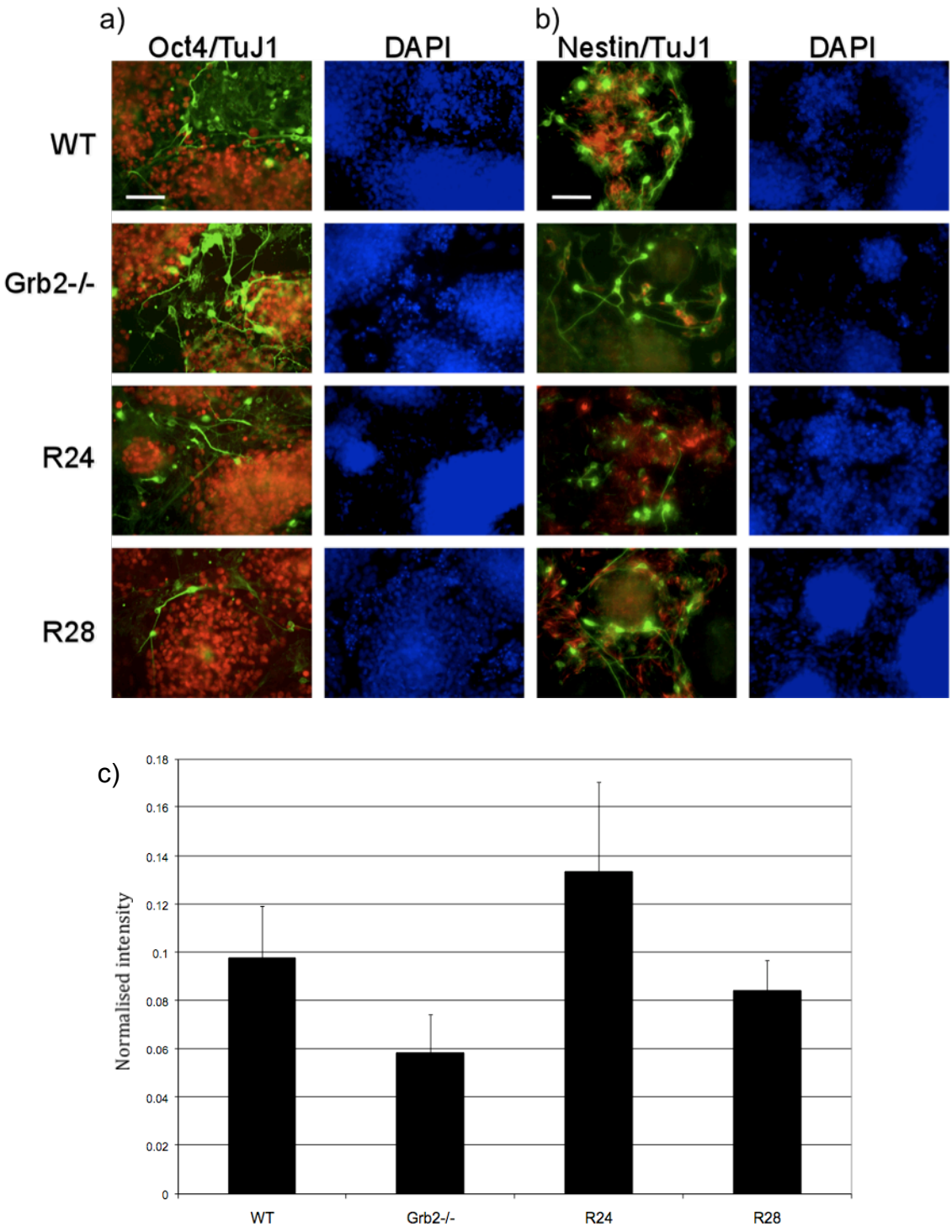
Figure 5.6 shows neural differentiation was almost eradicated in wild type and *Grb2* restored cell lines when cultured in the presence of 2μM PD03. On gelatin, wild type and *Grb2* restored lines remained *Oct4* positive after 14 days. Both *Grb2* restored lines produced neurons, but this was a rare occurrence. On laminin, wild type cells did not produce *β-III-tubulin* positive neurons. However, both *Grb2* restored lines produced a small number of cells bearing neural processes. These cell lines also exhibited poor adhesion to the laminin substrate, demonstrated by the holes in the cell monolayer.

Grb2$^{-/-}$ ES cells failed to expand efficiently on gelatin and laminin but neural differentiation was observed. A number of small *Oct4* positive colonies were observed and an increased number of *β-III-tubulin* neural processes were produced when plated on laminin.

5.2.2.5 *Grb2*$^{-/-}$ ES Cells Produce Fewer *Nestin* Positive Neuronal Precursors.

Proliferation of neural stem cells is dependent on FGF signalling (Kosaka *et al.*, 2006). As *Grb2* is a mediator of FGF signalling, the number of neural precursors was estimated through staining for neural precursor marker *Nestin*.

Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated on laminin at low density in N2B27 media in the presence of 1μM PD03 for 14 days. Cells were passaged on day 7. These conditions were considered optimal for capturing the maximum number of neural precursors in all cell lines. After 14 days cells were fixed and immunostained for *Oct4* and *β-III-tubulin* or *Nestin* and β-III-tubulin.


a)
Oct4/TuJ1
DAPI
b)
Nestin/TuJ1
DAPI
WT
Grb2-/-
R24
R28
c)
Normalised intensity
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
WT
Grb2-/-
R24
R28

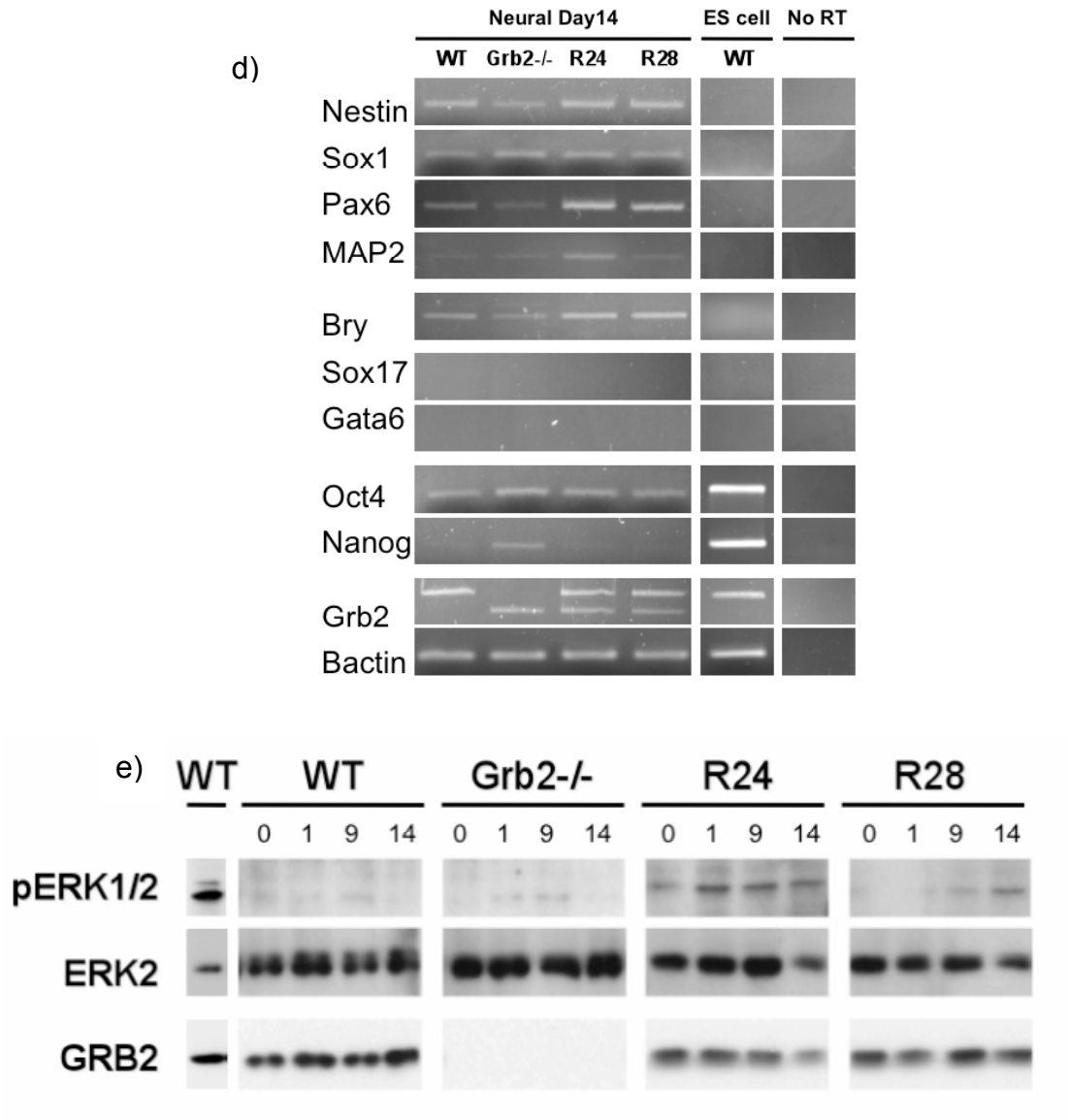


Figure 5.7 *Grb2*$^{-/-}$ ES cells produce fewer *Nestin* positive cells. Wild type, *Grb2*$^{-/-}$ ES cells and two *Grb2* restored ES cell lines were plated in N2B27+ 1μM PD0325901 on laminin coated plates at low density for 14 days. Cells were passaged at day 7. After 14 days, cells were fixed and immunostained. **a)** *Oct4* (Red), *β-III-tubulin* (Green), DAPI (Blue). **b)** *Nestin* (Red), *β-III-tubulin* (Green), DAPI (Blue). Scale bar – 50μm. **c)** Analysis of *Nestin* fluorescence intensity normalised to DAPI nuclear stain. Images of 3 random fields were analysed by Image J image analysis software. **d)** RT-PCR analysis of neural cultures. **e)** Western blot analysis of ERK phosphorylation at days 0, 1, 9 and 14 of neural differentiation. A wild type ES cell line cultured in N2B27-LB was utilised as a positive control.

After 14 days, all cell lines produced *β-III-tubulin* positive neuronal cells. *Oct4* positive cells were also observed (Figure 5.7a). Fewer *Grb2*$^{-/-}$ *Nestin*-positive cells were observed (Figure 5.7b) despite comparable numbers of fully differentiated neurons. The levels of *Nestin* were quantified via image

analysis. The fluorescence intensity of 3 fields of *Nestin*-stained and equivalent DAPI-stained neural cultures were measured using Image J image analysis software by normalising the signal to DAPI. Calculation of the intensity of *Nestin* signal suggested fewer *Nestin* positive cells were present in *Grb2*$^{-/-}$ cultures (Fig 5.7c).

In order to substantiate the claim that neural differentiation can occur in a *Grb2*-independent manner, RNA samples were collected from cells cultured on laminin in the presence of PD03 for 14 days. RNA samples were DNase treated and cDNA synthesised using the Invitrogen 'Superscript First-Strand cDNA Synthesis' kit for PCR analysis of gene expression. cDNA samples were assayed for neural markers *Nestin*, Sox1, Pax6 and MAP2 by PCR.

B-III-tubulin is expressed by most cell types and cannot be utilised as a neural marker using this technique. Its application in immunohistochemistry is widespread as a combination of strong *β-III-tubulin* expression and long cellular processes is unique to neurons. Microtubule-associated protein 2 (MAP2) is a structural protein responsible for the stabilisation of microtubules through cross-linking with intermediate filaments. MAP2 contains SH3 domains and has been identified as a direct binding partner of *Grb2* (Lim *et al.*, 2000). This may indicate a *Grb2*-mediated interaction between extracellular signals and regulation of the cytoskeleton. Expression of MAP2 is specific to neurons, making it a suitable marker for advanced neuronal differentiation using a PCR technique.

All cell lines expressed neural markers *Nestin*, Sox1, Pax6 and MAP2 (Figure 5.7d), indicating a range of neural precursors and differentiated neurons were present in the cultures.

Mesodermal marker Brachyury was expressed by all cell lines, suggesting differentiation was not restricted to the ectodermal lineage. Endodermal markers Gata6 and Sox17 were absent, indicating some restriction in

differentiation. ES cell marker *Oct4* was expressed in all cell lines, supporting the immunohistochemical data. Nanog expression was largely absent from wild type and *Grb2* restored cell lines but expressed in *Grb2*$^{-/-}$ cells.

5.2.2.6 Trace levels of ERK activation can be Detected in the Presence of PD0325901

To confirm that ERK signalling is abrogated in *Grb2*$^{-/-}$ cells in the presence of PD03, protein samples were taken on day 1, 9 and 14 of the laminin-based neural assay to be probed for ERK phosphorylation by Western blot. The photographic film was exposed to the chemiluminescent signal overnight to ensure capture of all signal. A positive control of wild type cells cultured in N2B27-LB was included. Figure 5.7e shows phosphorylated ERK can be detected, albeit at very low levels in comparison to wild type cells growing in N2B27-LB. These data suggest very low levels of ERK activation are sufficient to allow cells to undergo differentiation.

A study by Stavridis *et al.* (2007) showed only one hour of ERK activity is required for ES cells to become committed to differentiation. It was hypothesised that plating on laminin may cause a brief surge in ERK activity and this activity permits cells to commit to differentiation.

Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated on laminin-coated plastics in N2B27 containing 1μM PD03. Protein samples were collected at 4, 6, 8, 10 and 12 hours to be assayed for ERK phosphorylation by Western blot. The photographic film was exposed to the chemiluminescent signal overnight to ensure capture of all signal. Figure 5.5d shows low but detectable levels of ERK phosphorylation in all samples.

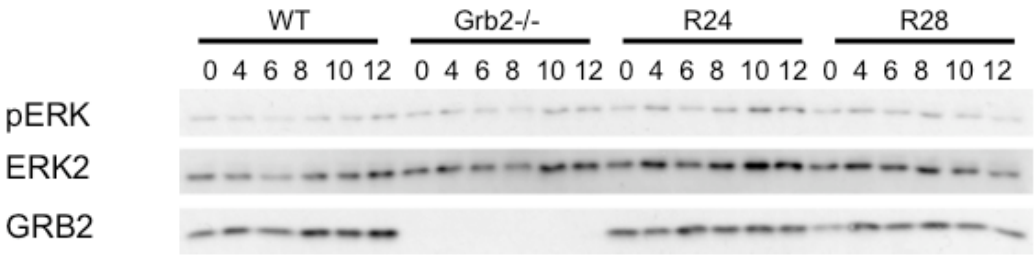


Figure 5.8 Western blot analysis of ERK phosphorylation upon plating on a laminin substrate in the presence of PD0325901. Wild type, *Grb2*$^{-/-}$ and two *Grb2* restored ES cell lines were plated on a laminin substrate in N2B27 + 1μM PD0325901. Protein samples were harvested at 4, 6, 8, 10 and 12 hours and assayed for ERK phosphorylation by Western blot using chemiluminescent detection. Signal emitted by the chemiluminescent reaction was collected by photographic film for 24 hours.

5.3 Discussion

This chapter demonstrates that *Grb2*$^{-/-}$ ES cells have a capacity to differentiate. Differentiation was detected in a LIF withdrawal monolayer assay, embryoid body culture assay and in a defined, monolayer differentiation assay.

5.3.1 *Grb2*$^{-/-}$ ES Cells Differentiate upon LIF Withdrawal

In the absence of LIF, wild type and *Grb2*$^{-/-}$ ES cells differentiated into large, flat alkaline phosphatase negative cells. Cells of a similar morphology are produced by ES cells plated in N2B27 containing BMP4 (Ying *et al.*, 2003; Kunath *et al.*, 2007). There is some evidence that these cells are early epidermal precursors (Harvey *et al.*, 2010).

5.3.2 *Grb2* is Required for Endodermal Differentiation

Experiments in this chapter show no expression of endoderm markers Gata6, Gata4 and Sox17 was detected in *Grb2*$^{-/-}$ EBs by RT-PCR, supporting previously published data. Cheng *et al.* (1998) reported a lack of endoderm after 2 days of EB differentiation by *Grb2*$^{-/-}$ ES cells. However, this was demonstrated using immunological staining for laminin and cytokeratin. These markers are non-specific, for example trophoblast cells also express cytokeratin (Sasagawa *et al.*, 1986) and this can be detected using the TROMA-1 antibody used in the aforementioned study. Laminin is also expressed at the 8 cell morula stage and in extraembryonic matricies. These findings were developed by Chazaud *et al.* (2006) when *Grb2*$^{-/-}$ embryos were shown to lack Gata6 expression via immunostaining, indicating defective primitive endoderm development at the blastocyst stage.

It was suggested that FGF-*Grb2*-MAPK signalling was responsible for this phenotype as both *Fgfr2*$^{-/-}$ and *FgfGF4*$^{-/-}$ embryos exhibit a similar restriction in endodermal differentiation (Arman *et al.*, 1998; Feldman *et al.*, 1995). This is supported by a recent study showing PD0325901 MEK inhibition prevents

primitive endoderm development in the ICM of developing blastocysts (Yamanaka *et al.*, 2010).

Differentiation of the definitive endoderm is distinct from primitive endoderm differentiation as it occurs later in the development of the embryo. Gata4, Gata6 and Sox17 also mark the definitive endoderm lineage. These markers were not detected in differentiating *Grb2*$^{-/-}$ EBs, indicating *Grb2* is required for both primitive and definitive endoderm differentiation. Like primitive endoderm, FGF signalling has been implicated in the regulation of definitive endoderm differentiation (Morrison *et al.*, 2008). However, the downstream effector of FGF signalling has not been confirmed. These data could suggest *Grb2* plays a similar role in both primitive and definitive endoderm differentiation.

5.3.3 *Grb2* is Required for the Formation of Beating Cardiomyocytes

Grb2$^{-/-}$ EBs failed to produce beating cardiomyocytes when plated on gelatin. Mesoderm markers Brachyury, Snail and Twist were detected upon EB formation, suggesting mesodermal differentiation is possible. However, while Brachyury is a marker of cells fated to make mesoderm, it may also mark cells with neural potential. Also, Snail and Twist have been detected in differentiating trophoblast cells that undergo an EMT immediately before implantation (Abell *et al.*, 2009). Indeed, trophoblast marker Cdx2 was expressed in the absence of *Grb2*. This suggests a larger panel of mesodermal markers should be studied in order to confirm mesoderm differentiation is *Grb2*-independent.

Another explanation for the lack of beating cardiomyocyte differentiation is the lack of GATA factor expression. Overexpression of Gata4 promotes the cardiac gene program in ES cells, resulting in a 10-fold increase in beating cardiomyocytes cells (Grepin *et al.*, 1997). Conversely, inhibition of Gata4 by RNAi inhibits cardiomyocyte development. It was reported that Gata4 deficient ES cells differentiated to the cardioblast stage before arresting and

undergoing apoptosis. A later study reported Gata4 deficient ES cells can form beating cardiomyocytes (Narita *et al.*, 1997) and it is thought that Gata6 can compensate for the loss of Gata4. This supports the observation that *Grb2*$^{-/-}$ EBs do not express Gata4 or 6 and fail to develop into beating cardiomyocytes.

5.3.4 *Grb2*$^{-/-}$ ES Cells Exhibit a Delay in Differentiation

Markers of differentiation were detected later in *Grb2*$^{-/-}$ differentiating EBs than in wild type or *Grb2* restored lines, while ES cell markers were more persistent. One explanation for this may be the abrogation of RTK-*Grb2*-MAPK signalling. *Grb2*-independent ERK activation is detected in the presence of serum. However, subtraction of *Grb2*-mediated RTK signalling such as FGF, IGF and LIF may result in a weaker ERK signal. A reduction in ERK activation may reduce the propensity for cells to undergo differentiation. This would result in a delay in leaving the naïve ES cell state without a defect in following the instruction to differentiate.

5.3.5 *Grb2* is Dispensable for Neural Differentiation.

Grb2$^{-/-}$ ES cells differentiate into terminally differentiated neurons in a defined monolayer culture system. Plating on a laminin substrate aided the formation of *Grb2*$^{-/-}$ neurons, however this effect was not observed in wild type or *Grb2* restored cells. This may be due to the poor attachment *Grb2*$^{-/-}$ colonies exhibit on a gelatin substrate as previously observed in *Grb2*$^{-/-}$ ES cells.

Neural differentiation occurs in the presence of a strong MEK inhibitor. In the presence of 1μM PD03, some *Oct4* positive cells persisted in the cultures indicating PD03 inhibits some differentiation. This is in agreement with published data (Burdon *et al*., 1999; Ying *et al*., 2008). However, all cell lines produced neurons after 14 days.

There was some evidence that *Grb2*$^{-/-}$ cells produced fewer *Nestin* precursors. This is in agreement with data presented by Kosaka *et al.* (2006)

wherein FGF signalling regulates the proliferation of neural stem cells. This reduced proliferation would result in a restricted number of precursors but would not inhibit the process of differentiation.

Increasing the level of PD03 to 2μM eradicated the appearance of all neurons in wild type cultures but a small number of these cells were produced by *Grb2* restored ES cell lines. This may indicate ERK signalling, and therefore differentiation has been shut down in these cells. However, at a concentration of 2μM, PD03 may confer non-specific inhibition of other kinases (Bain *et al.*, 2007).

Surprisingly, *Grb2*$^{-/-}$ ES cells formed neurons in the presence of PD03. It was assumed that abrogation of *Grb2* signalling combined with inhibition of MEK would extinguish all activation of ERK and differentiation would not occur. However, a highly exposed film showed weak ERK phosphorylation was detectable in *Grb2*$^{-/-}$ ES cells in the presence of MEK inhibition by Western blot. There are a number of potential explanations for this result.

The technical issue of antibody cross-reactivity with the non-phosphorylated form of ERK is unlikely to be responsible as levels of phosphorylated ERK oscillate between samples while levels of unphosphorylated ERK do not.

Another explanation may be incomplete inhibition by MEK inhibitor PD03. PD03 is a non-competitive inhibitor reported to be the most specific and potent inhibitor of MEK currently available (Bain *et al.*, 2007). The small molecule inhibitor has an affinity for a unique binding pocket adjacent to the ATP binding site of MEK (Ohren *et al.*, 2004). Inhibitor binding causes several conformational changes in the MEK molecule, resulting in a catalytically inactive protein. For these reasons it is unlikely that MEK signalling is possible in the presence of PD03.

Two studies implicate alternative signalling pathways in MEK-independent activation of ERK. Prolonged stimulation of NIH and Swiss 3T3 fibroblasts by PDGF results in PI3K and PKC-mediated ERK phosphorylation (Grammer and Blenis, 1997). Aksamitiene *et al.* (2010) also showed trace levels of ERK phosphorylation are detectable by Western blot in an EGF-stimulated breast cancer cell line in the presence of MEK inhibitor U0126. This phosphorylation was eradicated when MEK inhibition was combined with PI3K inhibitor Wortmannin or siRNA-mediated silencing of PI3K or AKT genes. It was suggested that PDZ-Binding Kinase/T-LAK cell-Originated Protein mediates this form of MEK independent ERK phosphorylation. However, inhibition of PI3K has been shown to enhance LIF-induced phosphorylation of ERK in ES cells (Paling *et al.*, 2004) so MEK-independent activation of ERK is unlikely to be mediated by this pathway. This leaves PKC as a potential candidate for weak ERK activation independent of MEK.

In an attempt to expand on these data, the neural assay was applied to *Erk2*$^{-/-}$ ES cells (Kunath *et al.*, 2007). These cells failed to produce *β-III-tubulin* positive neurons (see appendix), suggesting ERK is essential for differentiation. However, it was later discovered that the genetic approach used to delete *Erk2* from ES cells disrupted expression of a micro RNA cluster known to regulate neural differentiation. These complications render these data difficult to interpret.

This chapter demonstrates the novel finding that *Grb2* is not required for the differentiation of ES cells into some lineages. However, as no endodermal markers were detected, *Grb2*$^{-/-}$ ES cells cannot be considered truly pluripotent.

CHAPTER 6

Concluding Remarks

Embryonic stem cells are immortal and pluripotent. Advances in the understanding of ES cell biology have implications in our comprehension of early developmental processes and in the field of regenerative medicine. Experiments described in this thesis expand on current knowledge of signals regulating ES cell growth and differentiation in the context of the adapter protein *Grb2*.

Grb2$^{-/-}$ ES cells grow normally in serum-based media. However, under defined conditions, *Grb2*$^{-/-}$ ES cells grow in tight, poorly attached colonies and exhibit a decrease in proliferation compared to wild type cells. No increase in apoptosis was observed but it was demonstrated that *Grb2*$^{-/-}$ cells exhibit an abnormal cell cycle distribution under defined conditions. A large proportion of cells were seen in the G1 phase of the cell cycle. This is normal in somatic cells but not in ES cells. However, as *Grb2*$^{-/-}$ cells express ES cell marker *Nanog*, it is unlikely that this change in distribution was due to the onset of differentiation. One explanation for this change in distribution could be that the cell cycle is inhibited at the G1/S transition.

It was hypothesised that MAPK signalling was responsible for the abnormal cell cycle distribution. Indeed, no phosphorylation of ERK was detected in *Grb2*$^{-/-}$ ES cells in N2B27-LB. However, MAPK is not likely to be the missing signal that causes slow growth, as reinstating MAPK signalling via the addition of PMA or expression and activation of Raf-ER failed to increase the rate of proliferation. Cell cycle analysis of *Grb2*$^{-/-}$ ES cells under these conditions is required to confirm this idea.

Another possible explanation for the abnormal cell cycle distribution is that *Grb2* may mediate the degradation of cell cycle inhibitor p27. ES cells express low levels of this protein compared to somatic cells but the mechanism governing this level of expression is not known. Western blot

analysis of protein lysates will show whether the absence of *Grb2* results in high levels of this cell cycle regulator.

Serum contains a complex mixture of growth factors, fatty acids and adhesion molecules. This study suggests one or more of these factors are insufficient in N2B27-LB medium, and *Grb2*-mediated signalling enables efficient growth in their absence.

As both FGF and IGF signalling did not activate the MAPK pathway, it is unlikely that growth factor signalling causes the differences in behaviour between *Grb2*$^{-/-}$ ES cells in serum-based and serum-free media. This is supported by the failure of both PMA and Raf-ER to restore efficient growth of *Grb2*$^{-/-}$ ES cells in serum-free medium, despite successful activation of MAPK signalling. However, it appears activation of Raf-ER in *Grb2*$^{-/-}$ ES cells changes their adhesive properties, which may suggest a role for *Grb2*-mediated MAPK signalling in ES cell adhesion.

Grb2$^{-/-}$ ES cell colonies adhere poorly to a gelatin substrate. Interestingly, there was no defect in the initial adhesion of *Grb2*$^{-/-}$ cells. This suggests that a secondary adhesion event mediated by *Grb2* may be responsible for colony attachment to this substrate. *Grb2* is involved with linking integrins and the cytoskeleton to intracellular signals and may be required for the assembly of the complexes that form at focal adhesions.

Plating on laminin and fibronectin substrates restored colony adhesion to *Grb2*$^{-/-}$ ES cells. These substrates interact with an alternative complement of integrin subunit heterodimers and this may account for the differences in colony adhesion observed. This can be investigated using antibodies or blocking peptides specific to integrins to recapitulate the changes in attachment exhibited by *Grb2*$^{-/-}$ ES cells in N2B27-LB medium.

It is likely that one or more cell attachment molecules (CAMs) that are present in serum are responsible for augmenting ES cell growth and adhesion in the absence of *Grb2*. The specific molecule(s) could be elucidated experimentally by incubating the cells with CAM-specific

antibodies or blocking peptides. Blocking the function of these CAMs may recreate the colony morphology presented by *Grb2*$^{-/-}$ ES cells in N2B27-LB medium.

Expression and activation of Raf-ER also rescued colony adhesion on a gelatin substrate. This indicates *Grb2*-Raf-mediated signalling is required for efficient colony adherence. It is likely that MAPK is the downstream effector of this signal. Application of MEK inhibitor PD032901 to the Raf-ER system will test this idea. If MAPK signalling is critical for effective colony adherence, inhibition of MEK will reverse the Raf-ER-augmented improvement in adhesion.

Interestingly, despite the changes in morphology, plating on laminin or fibronectin substrates or activation of Raf-ER did not improve the proliferation rate of *Grb2*$^{-/-}$ ES cells in N2B27-LB. This suggests that these processes of colony adhesion and proliferation are not linked.

Grb2$^{-/-}$ ES cells exhibited an increase in phosphorylation of AKT at serine residue 471 in response to IGF1, in a manner similar to wild type cells. This suggests IGF-PI3K signalling is not mediated by *Grb2*.

No induction of phosphorylation of AKT was observed in response to FGF2 in *Grb2* or wild type ES cells. Therefore, no conclusion can be made about the role *Grb2* plays in FGF-mediated PI3K signalling. Expanding this study to include a time course of FGF induction may reveal AKT phosphorylation at a later time point.

Phosphorylation of ERK1/2 at residues thr202/tyr204 respectively was not detected in *Grb2*$^{-/-}$ ES cells in response to IGF or FGF induction. Furthermore, no phosphorylation of ERK was detected in cells cultured in N2B27-LB. This lead to the hypothesis that MAPK signalling was required for efficient growth in this medium. However, no improvement in proliferation rate was observed upon activation of ERK signal through addition of PMA or expression and activation of Raf-ER. This suggests MAPK signalling does not augment efficient growth in N2B27-LB.

Experiments in chapter 5 showed a very low level of ERK phosphorylation was present in *Grb2*$^{-/-}$ ES cells in N2B27 medium, even in the presence of MEK inhibitor PD03. This suggests that MAPK signalling is not eliminated in *Grb2*$^{-/-}$ cells and may support the data suggesting MAPK is not the downstream factor regulating growth.

Inhibition of GSK3β improves growth of cells under FGFR and MEK inhibition (Ying *et al.*, 2008). As no ERK phosphorylation was detected in *Grb2*$^{-/-}$ ES cells in N2B27-LB, it was hypothesised that growth would be restored upon addition of GSK3β inhibitor CHR99021. GSK3β inhibition did not improve growth of *Grb2*$^{-/-}$ cells. This indicates *Grb2*$^{-/-}$ ES cells are not equivalent to cells under FGFR and MEK inhibition.

To investigate how GSK3β inhibition failed to improve growth, analysis of GSK3β signalling should be carried out, including ascertaining the level of phosphorylated GSK3β in wild type and *Grb2*$^{-/-}$ ES cells in N2B27-LB and whether any effect can be detected on downstream components of the GSK3β signal. This may include assaying the level of phosphorylated and unphosphorylated β-catenin and the location of this molecule in the cell. High levels of phosphorylated GSK3β should result in high levels of unphosphorylated β-catenin in the nucleus and may indicate that this pathway is misregulated in *Grb2*$^{-/-}$ ES cells.

Grb2 may be a useful target for therapeutic intervention of cancer. Giubellino *et al.* (2007) stated *Grb2* plays a role in metastasis. This thesis supports these findings; demonstrating deletion of *Grb2* changes the adhesive properties of ES cells, although this is only evident under serum-free conditions. Of particular interest is the combination of *Grb2* and GSK3β inhibition. Data presented in this thesis may suggest that application of this dual inhibition to the *in vivo* models described by Giubellino *et al.* (2007) may impact significantly on the proliferation and migration of cancerous cells.

Grb2$^{-/-}$ ES cells were subjected to a number of different differentiation protocols and demonstrated a capacity to differentiate, with some restrictions.

Grb2$^{-/-}$ ES cells demonstrated the ability to differentiate into advanced cell types of the ectoderm lineage. Additional MAPK inhibition via PD0325901 did not prevent differentiation in N2B27. Very low levels of ERK phosphorylation were detected in *Grb2*$^{-/-}$ cells under MEK inhibition, indicating differentiation was not MAPK-independent. This does suggest that very low levels of ERK activation are permissive for exiting self-renewal and differentiating into neurons. Differentiation is asynchronous and many cells remain undifferentiated in these cultures. This suggests a stochastic element in the mechanism of *Grb2*-independent, low MAPK differentiation. This may also infer *Grb2*-deficient cultures under MEK inhibition exhibit a low level of heterogeneity. This may be a result of a novel autocrine signal or an effect of cell density that is inherent in *in vitro* culture.

Mesoderm markers *Brachyury*, *Snail* and *Twist* were expressed in *Grb2*$^{-/-}$ EB cultures. However, these markers may not be sufficient to confirm the presence of mesoderm in these cultures. Investigating the expression of an additional marker, such as Msh homeobox 1 (*Msx1*) may help elucidate whether mesodermal differentiation is possible. Indeed, beating cardiomyocytes were not produced by differentiating *Grb2*$^{-/-}$ ES cells, suggesting *Grb2* is required for differentiation into some lineages of the mesoderm. A lack of beating cardiomyocytes may be due to the abrogated expression of Gata factors 4 and 6 in *Grb2*$^{-/-}$ EBs. Careful analysis of the markers of cardiomyocyte differentiation could identify the stage at which *Grb2* is required for these cells to form.

Grb2-mediated signalling is required for differentiation into the endoderm lineage. This expands upon published data which describes short-term *in vitro* assays and a limited set of markers (Cheng *et al.*, 1998; Chazaud *et al.*, 2006). *Grb2*$^{-/-}$ embryoid bodies do not express endoderm marker Gata6 after 10 days in culture. Markers Gata4 and Sox17 were also absent. The

limitation of *Grb2*$^{-/-}$ ES cells to produce cells of the endoderm lineage suggests they lack true pluripotency.

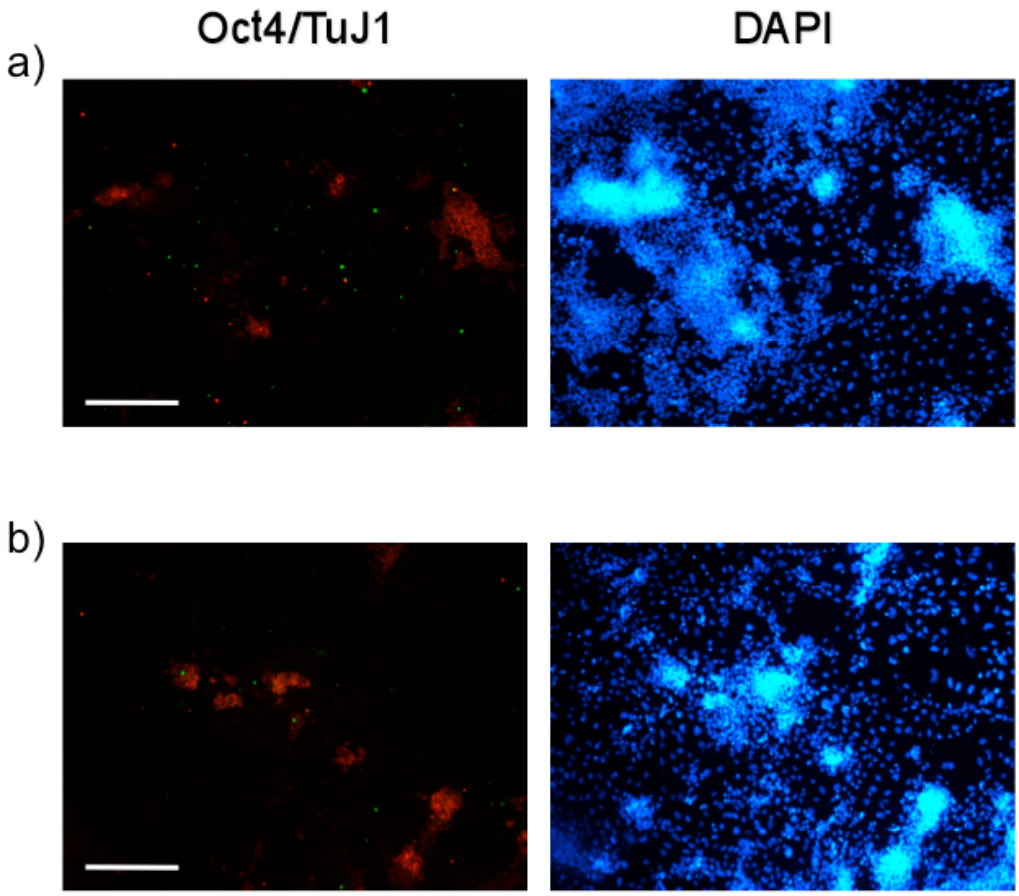
As *Grb2*$^{-/-}$ ES cells exhibit restricted differentiation, this limitation could be used as an advantage in controlling the generation of ES cell-derived tissue. Selective inhibition of *Grb2* can be achieved through the application of peptide analogs (Lung *et al.*, 2002) or small molecules such as CGP85793 (Gay *et al.*, 1999). Utilisation of these compounds may increase the homogeneity of differentiation cultures by eliminating cell fates such as the endoderm and cardiac lineages, but at the cost of efficient growth.

The ground state hypothesis describes a state in which an ES cell is unaffected by any process of differentiation. It essentially concerns the status of ES cells in relation to their pluripotency. *Grb2*$^{-/-}$ ES cells are often used to support this hypothesis as they are considered to have limited differentiation potential due to their lack of MAPK signalling (Cheng *et al*., 1998), the key signal that causes an ES cell to leave the naïve ground state (Kunath *et al*., 2007; Ying *et al.,* 2008). This thesis shows *Grb2*$^{-/-}$ ES cells can readily leave the ground state under MEK inhibition, which severely reduces, but does not eradicate MAPK signalling. This suggests that MAPK suppression by this method is not sufficient to preserve ES cells in a naïve ground state.

Furthermore, the ground state hypothesis does not yet take into consideration other cellular processes such as cell proliferation and adhesion, which are critical for the survival of any cell *in vivo* and *in vitro*.

The experiments presented in thesis describe a novel contribution of *Grb2* in growth and adhesion and expands upon current knowledge of its role in differentiation. Further investigation into the processes mediated by *Grb2* in ES cells will provide a valuable insight into the mechanisms governing ES cell biology and the early events of embryonic development.

APPENDIX



ERK2-/- ES cells do not produce neurons. ERK2-/- ES cells (Kunath *et al*., 2007) were plated in N2B27 on a) gelatin or b) laminin coated plates at low density for 14 days. Cells were passaged at day 7. After 14 days, cells were fixed and immunostained for ES cell marker Oct4 (Red) and neural marker beta-III-tubulin (Green). Nuclei were DAPI stained (Blue). Scale bar - 200μm

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